Biogenic Amines in Wine

Gene transcription as a tool for selection of *Oenococcus oeni* starter strains

Ana Paula Gomes Marques

Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

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"A man´s friendships are one of the best measures of his worth"

Charles Darwin

To my dear family

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SUMMARY

Wine is a complex environment where the microbial flora can have both a positive (e.g. malolactic fermentation) and a negative (e.g. production of biogenic amines) impact on the quality of wine.

Biogenic amines are nitrogenous compounds of low molecular weight that can be found in fermented food and beverages, including wine. Biogenic amines are formed primarily by decarboxylation of the corresponding amino acids by microorganisms through substrate-specific decarboxylases. Some authors consider the presence of biogenic amines to be a fundamental parameter for detriment of alcoholic beverages. These compounds can have adverse health effects on sensitive individuals at high concentrations.

The role of microorganisms in wine involves two important fermentation processes, the alcoholic fermentation conducted by yeasts and the malolactic fermentation conducted by lactic acid bacteria. The malolactic fermentation refers to the conversion of L-malic acid into L-lactic acid and CO_2, catalysed by the malolactic enzyme. This mechanism contributes for deacidification, bacterial stability and flavour changes in wine. *Oenococcus oeni* is the leading lactic acid bacteria responsible for malolactic fermentation, mainly due to its adaptability to such a chemically harsh wine environmental (low pH and high ethanol concentrations).

The two main goals of this PhD thesis were to contribute to the: (1) increase of knowledge of biogenic amines occurrence in wine; (2) oenological characterization and selection of Portuguese autochthonous *O. oeni* strains, isolated from different winemaking regions, to be used as malolactic starters on the wine industry.

The study of the influence of oenological factors on the production of biogenic amines in wine revealed that winemaking region, grape varieties, commercial malolactic starters and wine storage on lees affect the presence and the amounts of biogenic amines (mainly tyramine) in wine. Also, fungal metabolic

activity could contribute for the biogenic amines formation (especially isoamylamine).

The work carried out in this thesis allowed the development of a new molecular method, based on the amplification of 16S rRNA gene with universal primers, followed by restriction with the endonuclease FseI. This method ensures the rapid and reliable detection of *O. oeni* in wine samples during winemaking surveillance and wine quality control. M13-PCR fingerprinting analysis revealed a high level of intraspecific genomic diversity in 121 Portuguese autochthonous *O. oeni* strains isolated from wines of three different winemaking regions. This diversity could be partitioned according to the geographical origin of the isolates. Therefore, M13-PCR fingerprint analysis may be an appropriate methodology to study the *O. oeni* ecology of wine during malolactic fermentation as well as to trace new malolactic starter cultures and evaluate their dominance over the native microbiota.

Oenological characterization of *O. oeni* strains obtained from wines collected in three different winemaking regions of Portugal was performed through the screening of malolactic and β-glucosidase activities, and production of biogenic amines and ethyl carbamate precursors. In order to assess functional behaviour, *O. oeni* strains were tested for their capacity to grow under different conditions in “synthetic wine” and culture media, as an additional criterion to select the most promising strains to be candidates as starters in winemaking processes. Our results emphasize the importance of the characterization of *O. oeni* strains regarding the production of enzymes and undesirable compounds as criteria for the selection of malolactic starters. By applying multivariate statistics to data obtained from growth behaviour of Portuguese strains under different conditions, a strain from each winemaking region of Portugal was selected as the most suitable regional O. *oeni* strain for malolactic fermentation accomplishment in wines obtained from the same region.

The RNA fingerprinting analysis (RAP-PCR) of *O. oeni* strains under wine conditions displays that starter strains showed more constrained and limited transcription profiles, whereas a high variation on the distribution of the transcription profiles was observed for the regional strains in each wine. Although the wine matrix appears to be the dominant factor in gene expression, the behaviour of each strain seems to be dependent on its gene pool. Thus, this behaviour may be associated with differential gene expression pools induced by differences in the wine matrix. RAP-PCR could be a useful technique for a preliminary investigation of strain behaviour under different wine environmental conditions. According to our results, this technique could be applied in field studies to monitor differential patterns of global gene expression and to select markers for the surveillance of starter performance in winemaking, as well as for quality and safety control.

The study of the effect of malic acid, pH, ethanol and sulphur dioxide on *O. oeni* malolactic enzyme (*mleA*) gene and arginine deiminase (*arcAC*) gene cluster showed down-regulation for low concentrations of malic acid and low pH, and up-regulation for high ethanol content. High levels of sulphur dioxide reduced *mle*A and increased *arc*AC expression. The outcomes observed are here considered as adaptive responses to compensate possible inhibitory effects of environmental conditions on the physiological activities of bacterial cells.

RESUMO

O vinho é um ambiente complexo, onde a flora microbiana pode ter um impacto positivo na sua qualidade, por exemplo através da fermentação maloláctica, e/ou negativo, como é o caso da produção de aminas biogénicas. As aminas biogénicas são compostos nitrogenados de baixo peso molecular que podem ser encontradas em alimentos e bebidas, incluindo o vinho. Estas bases orgânicas são essencialmente formadas a partir da descarboxilação dos aminoácidos precursores, pela acção de enzimas microbianas. A presença de aminas biogénicas é considerada, por alguns autores, um parâmetro fundamental para a qualidade final de bebidas fermentadas. Estes compostos podem ter efeitos adversos na saúde de indivíduos sensíveis quando presentes em concentrações elevadas.

O papel dos microorganismos no vinho envolve dois importantes processos de fermentação, a fermentação alcoólica realizada por leveduras e a fermentação maloláctica (FML), conduzida por bactérias do ácido láctico. A fermentação maloláctica corresponde à conversão de ácido L-málico em ácido L-láctico e dióxido de carbono pela acção da enzima maloláctica. Este mecanismo contribui para a desacidificação e estabilidade bacteriana do vinho, bem com para as suas propriedades organolépticas. *Oenococcus oeni* é uma bactéria do ácido láctico com interesse económico na produção de vinho, sendo a espécie preferencialmente seleccionada para a fermentação maloláctica, pois possui adaptabilidade às características complexas e austeras do vinho (pH baixo e teores elevados de etanol).

Os dois principais objectivos desta tese de doutoramento foram contribuir para: (1) o incremento do conhecimento da ocorrência de aminas biogénicas no vinho; (2) a caracterização enológica e selecção de estirpes Portuguesas autóctones de *O. oeni*, de diferentes regiões geográficas, para serem aplicadas como “starters” da FML na indústria vitivinícola.

O estudo da influência de factores enológicos sobre a produção de aminas biogénicas no vinho revelou que a região vitivinícola, as castas, os “starters” comerciais para a FML e o armazenamento do vinho sobre borras, influencia a presença e os valores de aminas biogénicas, em especial de tiramina, no vinho. Os resultados obtidos permitiram verificar que a actividade metabólica de fungos também pode contribuir para a formação de aminas biogénicas, com especial relevância para a isoamilamina.

O trabalho realizado nesta tese possibilitou o desenvolvimento de um novo método molecular que permite a detecção de *O. oeni* no vinho, de um modo rápido, para ser aplicado no controlo de qualidade do processo de vinificação. Este método consiste na amplificação do gene 16S rRNA utilizando primers universais e posterior digestão com a enzima de restrição FseI. A análise M13-PCR “fingerprinting” revelou um elevado nível de diversidade genómica intra-específica nos 121 isolados de *O. oeni* provenientes de vinhos de três regiões vitivinícolas Portuguesas. Esta diversidade permite verificar um agrupamento de clusters genómicos de acordo com a origem geográfica dos isolados. Sendo assim, a análise M13-PCR "fingerprinting" demonstrou ser uma metodologia adequada para estudar a ecologia de *O. oeni* durante a FML, bem como para seleccionar novas culturas “starters” e avaliar a sua dominância sobre a microflora endógena.

A caracterização enológica de isolados de *O. oeni* de vinhos de três regiões vitivinícolas de Portugal foi efectuada através da pesquisa das actividades maloláctica e β-glicosidase, bem como da produção de aminas biogénicas e de precursores de carbamato de etilo. Com o objectivo de avaliar o comportamento funcional, e como critério adicional para seleccionar as melhores estirpes de *O. oeni* candidatas a "starters" no processo de vinificação, 51 estirpes de *O. oeni* foram testadas para a sua capacidade de crescimento em diferentes condições em "vinho sintético" e meio de cultura. Os resultados obtidos reforçam a importância da caracterização de estirpes de *O. oeni*, no que se refere à produção de enzimas e compostos

indesejáveis como critérios para a selecção de "starters" para a FML. A aplicação de estatística multivariada aos resultados obtidos a partir do comportamento de crescimento de estirpes autóctones, em diferentes condições, permitiu a selecção de uma estirpe de *O. oeni* com potencial aplicação na FML, por cada uma das regiões vitivinícolas abrangida pelo estudo.

A análise de RNA "fingerprinting" de estirpes de *O. oeni* em diferentes vinhos demonstrou que as “starters” comerciais possuem perfis de transcrição mais restritos e limitados, enquanto que para as estirpes autóctones foi observada uma elevada variação na distribuição dos perfis de transcrição em cada vinho analisado. Apesar da matriz do vinho ser um factor dominante na expressão génica, o comportamento de cada estirpe é dependente da sua *pool* de genes. Assim sendo, este comportamento pode estar associado a diferentes pools de expressão génica cuja expressão pode ser induzida pelas diversas matrizes de vinhos.

Os resultados da presente investigação indicam que a RAP-PCR (do Inglês *Random Arbitrarily Primed PCR*) pode ser uma metodologia com potencial aplicação na investigação preliminar do comportamento de estirpes em diferentes vinhos, podendo ser utilizada na análise global da expressão génica e na selecção de marcadores para a monitorização da performance de “starters” no processo de vinificação, bem como no controlo da qualidade e segurança.

O estudo do efeito do ácido málico, do pH, do etanol e do dióxido de enxofre (SO_2) na expressão do gene da enzima maloláctica (*mleA*) e no cluster da arginina deiminase (*arcAC*) em *O. oeni* demonstrou uma sub-expressão para baixas concentrações de ácido málico e pH baixo, bem como uma sobre-expressão para teores de etanol elevados. Por outro lado, elevadas concentrações de SO_2 conduzem a um aumento da expressão do cluster *arcAC* e uma redução da expressão do gene *mleA*. Os resultados observados podem ser interpretados como uma forma de resposta adaptativa aos efeitos

inibitórios na actividade fisiológica das células bacterianas às condições ambientais.

AIMS

General Aim:

The work developed throughout this thesis aimed to study the influence of oenological factors on the production and on the amounts of biogenic amines in wines and the oenological characterization and selection of suitable Portuguese autochthonous *Oenococcus oeni* strains to be used as malolactic starters in the wine industry.

Specific Aims:

To attain these goals, the following specific aims were pursued:

1. Study of the effect of some oenological factors (winemaking region, grape variety, anti-fungal treatment of grapes, fermentation activators, malolactic starters and storage on lees) from the point of view of their influence on the content of biogenic amines in wines;

2. Development of a new molecular method for the identification of *O. oeni* strains and their specific detection in wine;

3. Analysis of the genomic diversity of *O. oeni* strains isolated from three different winemaking regions of Portugal;

4. Assessment of oenological characteristics of Portuguese autochthonous *O. oeni* strains (phenotypic and molecular screening of enzymatic activities and growth analysis under different environmental conditions) for the selection of malolactic starters with high levels of malolactic and β-glucosidase activity and lack of or low capacity to produce biogenic amines and ethyl carbamate precursors;

5. Evaluation of global transcriptional profiles of *O. oeni* strains under wine environmental conditions;

6. Study of the influence of malic acid and pH, ethanol and sulphur dioxide stresses on the expression of the malolactic enzyme gene and arginine deiminase gene cluster in a specific *O. oeni* strain.

THESIS PLANNING

The present PhD thesis is organized into five different chapters.

In Chapter I a general introduction to the subject of the thesis is presented, including a literature review focusing the identification, the taxonomy, and the ecology of wine lactic acid bacteria, especially *Oenococcus oeni* and their oenological importance.

Chapter II presents the results about the influence of oenological factors that could contribute for the production and presence of biogenic amines in wines.

The first part of Chapter III is dedicated to the development of a new molecular method for the identification of *O. oeni* and its specific detection in wine. The second part of Chapter III focuses the isolation and the identification of *O. oeni* strains from wines of different winemaking regions of Portugal and also the evaluation of the genetic diversity of this collection of *O. oeni* strains. The third part of Chapter III covers the selection of Portuguese autochthonous *O. oeni* strains to be used as malolactic starters on wine industry.

The first part of Chapter IV aims to analyze the transcriptome profile of Portuguese autochthonous *O. oeni* strains and malolactic starters in wines from different winemaking regions of Portugal. The second part of Chapter IV focus on the study of the response of malolactic enzyme gene and arginine deiminase gene cluster in a specific *O. oeni* strain under the effect of different wine stresses.

The discussion and future perspectives of the thesis are presented in Chapter V.

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CHAPTER 1

INTRODUCTION

1.1 LACTIC ACID BACTERIA

1.1.1 General characterization

Lactic acid bacteria (LAB)[1] belong to the phylum "Firmicutes", class "Bacilli", order "Lactobacillales" and are grouped in six families ("Lactobacillaceae", "Aerococcaceae", "Carnobacteriaceae", "Enterococcaceae", "Leuconostocaceae" and "Streptococcaceae") (Axelsson, 2004; Makarova and Koonin, 2007). The principal LAB associated with fermented food and beverage are grouped in genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Stiles and Holzapfel, 1997; Ercolini *et al.*, 2001; Holzapfel *et al.*, 2001; Axelsson, 2004; Jay *et al*., 2005).

The taxonomy of LAB based on comparative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxa generated on the basis on phenotypic features are not supported by the phylogenetic relations (Gevers *et al.*, 2001; Holzapfel *et al.*, 2001).

Lactic acid bacteria are a metabolically and physiologically heterogeneous family of gram-positive and strictly fermentative rods or cocci, usually non-motile, catalase negative in the absence of porphorinoids, nonsporing, aerotolerant, acid tolerant and organotrophic. These bacteria have complex nutritional requirements and most of the species have multiple needs of amino acids and vitamins. They produce lactic acid as the primary metabolite of sugar metabolism (Davis *et al.*, 1985, 1988; Lonvaud-Funel, 1999; Carr *et al.*, 2002; Liu, 2002; Axelsson, 2004; Liu *et al*., 2005).

This group of bacteria can be found in foods (milk, cheese, yoghurt, fermented meat products, and sourdough), beverages (beer, and wine), soil, water, plants (fruits, vegetables, cereal grains, manure, sewage, silage) and

[1] In this thesis, lactic acid bacteria are considered in the most current *sensu strictu* that only includes members of the "Lactobacillales".

microbiota of mucous membranes (intestines, mouth and vagina) of both humans and animals (Aukrust and Blom, 1992; Fujisawa and Mitsuoka, 1996; Gobbetti and Corsetti, 1997; Boris *et al.*, 1998; Ocaña *et al.*, 1999; Lonvaud-Funel, 2001; Martín *et al.*, 2003; Sbordone and Bortolaia, 2003; Jay *et al*., 2005).

Lactic acid bacteria are widely used as starters to achieve favourable changes in texture, flavour and also to contribute for the nutritional qualities and for the prevention of food spoilage, as these microbes are responsible for the initial acidification of the raw material through the production of lactic acid (Caplice and Fitzgerald, 1999; Liu, 2003). LAB can also produce bioactive molecules such as acetaldehyde, hydrogen peroxide, carbon dioxide, diacetyl, polysaccharides, and ethanol (Caplice and Fitzgerald, 1999; de Vuyst and Degeest, 1999). Many strains also produce bacteriocins or bacteriocin-like molecules, and some strains produce specific low-molecular-mass inhibitory compounds (reuterin, reutericyclin). Some LAB strains display interesting probiotic properties inhibiting gastrointestinal pathogenic bacteria and also can reveal antifungal activity by the production of specific organic acids (phenyllactic acid) and/or cyclic dipeptides (Schillinger and Lücke, 1989; Bredholt *et al*., 2001; Budde *et al*., 2003; Jacobsen *et al*., 2003; Vermeiren *et al*., 2004, Brillet *et al*., 2005).

The worldwide use of LAB in the production of foods and beverage has increase significantly the scientific interest of the academic researchers and industrial producers over the last decades. Several major advances have been achieved, leading to a new range of applications. Recently, those scientific breakthroughs culminated in the genomic caracterization of different LAB species (Bolotin *et al.*, 2001, 2004; Pridmore *et al.*, 2004; Altermann *et al.*, 2005; Liu *et al*., 2005; Makarova *et al.*, 2006; van de Guchte *et al.*, 2006; Callanan *et al.*, 2008; Durmaz *et al.*, 2008).

1.1.1.2 Metabolism of sugars

Lactic acid bacteria are chemotrophic, therefore the energy required for their metabolism is provided by the oxidation of chemical compounds. The oxidation of sugars (*e.g.* hexoses and pentoses) constitutes the main energy-producing pathway essential for bacterial growth. In LAB, fermentation is the way for the assimilation of sugars. For a specific LAB species, the type of sugar fermented and the environmental conditions (presence of electron acceptors, pH, etc.) modify the energy yield and the nature of the final products (Fooks *et al.*, 1999; Caplice and Fitzgerald, 1999; Kuipers *et al.*, 2000; Jay *et al*., 2005; Ribéreau-Gayon *et al*., 2006).

1.1.1.2.1 Homofermentative metabolism of hexoses

In the homofermentative pathway, also known as Embden-Meyerhoff-Parnas (EMP) mechanism, the bacteria convert almost all hexoses, especially glucose (more than 90%), into lactic acid. A first phase containing all the reactions of glycolysis that lead from hexose to pyruvate. During this stage, the oxidation reaction takes place generating the reduced coenzyme NADH + H^+. The second phase characterizes lactic fermentation. The reduced coenzyme is oxidized into NAD^+ during the reduction of pyruvate into lactic acid. Representative homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, and group I lactobacilli (Caplice and Fitzgerald, 1999; Kuipers *et al.*, 2000; Jay *et al*., 2005; Ribéreau-Gayon *et al*., 2006).

1.1.1.2.2 Heterofermentative metabolism of hexoses

Bacteria with heterofermentative metabolism use the pentose phosphate pathway, alternatively referred to as the pentose phosphoketolase pathway. One mole of glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP is

further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. In theory, end-products (including ATP) are produced in equimolar quantities from the catabolism of one mole of glucose. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella*, and group III lactobacilli (Caplice and Fitzgerald, 1999; Kuipers *et al.*, 2000; Jay *et al*., 2005; Ribéreau-Gayon *et al*., 2006).

1.2 WINE LACTIC ACID BACTERIA

1.2.1 Taxonomy

Lactic acid bacteria are naturally found on grape berries surface, stems, leaves, soil, and winery equipment. However, as wine is an extremely selective medium, only a few types of LAB are able to grow in this environment. An important factor for the wine quality is the ability of LAB to metabolise malic acid into lactic acid in order to reduce wine acidity. Nevertheless, besides this positive aspect, several wine compounds (sugars, tartaric acid, and glycerol) can also be metabolised inducing wine spoilage (bitterness, mousiness, ropiness) (Lafon-Lafourcade *et al.*, 1983; Fleet, 1993).
The LAB isolated from grape, must or wine belongs to two families. The family "Lactobacillaceae" is represented by the genus *Lactobacillus* and and the family "Streptococcaceae" is represented by the genera *Oenococcus* and *Pediococcus (*Fugelsang and Edwards, 2007; Pozo-Bayón et al., 2009). Table 1 shows some characteristics of wine LAB genera. The main lactic acid bacteria species found in grape musts and wines are listed in Table 2. These bacteria are generally microaerophilic, require carbohydrates, and culture media must be supplied with amino acids and vitamins in order to allow their proliferation (Wibowo *et al.*, 1985; Fugelsang and Edwards, 2007).

Table 1 – Some characteristics of LAB genera associated with wine (adapted from Gindreau *et al.*, 2001; du Plessis *et al.*, 2004; Bartowsky, 2005; Björkroth and Holzapfel, 2006; Fugelsang and Edwards, 2007).

Genus	Characteristic				
	Cell morphology and arrangement	Carbohydrate fermentation	CO_2 from glucose	Lactic acid isomer	DNA G+C content
Lactobacillus	Rods, coccobacilli cells single or in chains	Homo- or heterofermentative facultatively heterofermentative	+ and -	D, L, DL	36 to 46%
Leuconostoc	Spherical or lenticular cells in pairs or chains	Heterofermentative	+	D	38 to 44%
Oenococcus	Spherical or lenticular cells in pairs or chains	Heterofermentative	+	D	38 to 44%
Pediococcus	Spherical cells, pairs or tetrads	Homofermentative or facultatively heterofermentative[a]	-	DL, L	34 to 42%
Weissella	Spherical, lenticular, irregular cells	Heterofermentative	+	D, DL	38 to 40%

a - Facultatively heterofermentative species: *P. pentosaceus*, P. *acidilactici*, *P. claussenii*.

Symbols: +, positive reaction; -, negative reaction.

Table 2 – Main lactic acid bacteria species isolated from musts and wines (Pozo-Bayón *et al*., 2009).

Genus	Species
Lactobacillus	*L. brevis, L. casei, L. cellobiosus, L. fermentum, L. hilgardii, L. kefiri, L. kunkeei, L. lindneri, L. mali, L. nagelii, L. paracasei, L. plantarum, L. trichodes, L. vermiforme, L. vini, L. zeae*
Pediococcus	*P. pentosaceus*, *P. damnosus*, *P. parvulus*
Leuconostoc	*L. mesenteroides*
Oenococcus	*O. oeni*

In this work, special focus will be dedicated to genera *Oenococcus*, since the work presented in this thesis is based on studies in *Oenococcus oeni* strains, isolated from wines of different regions of Portugal.

1.2.1.1 Genus *Oenococcus*

The species of the genus *Oenococcus* are spherical or lenticular cells in pairs or chains, nonmotile, asporogenous, chemoorganotrophic, facultatively anaerobic, nonproteolytic, nonhemolytic, do not present cytochromes, nitrates are not reduced and indole is not formed (Ribéreau-Gayon *et al*., 2006; Fugelsang and Edwards, 2007). *Oenococcus* has been separated from the genus *Leuconostoc* by 16S rRNA sequence analysis in 1995 by Dicks *et al.* (1995). This genus groups only two species, *Oenococcus oeni* and *O. kitahareae* (Endo and Okada, 2006). The last one has been isolated from a composting distilled shochu residue. In *O. kitahareae*, L-malate is not decarboxylated to L-lactate and CO_2 in the presence of fermentable sugars. Cells do not grow below pH 4.5 and in 10% ethanol. Growth is not stimulated by tomato juice. The DNA G+C content ranges from 41 to 43 mol% (Endo and Okada, 2006).

1.2.1.1.1 Oenococcus oeni

O. oeni occurs naturally in wine and related habitats (vineyards and wineries) and is characterized by its peculiar acidophilic nature and growth in media containing relative high ethanol levels (> 10%) (Garvie, 1986).

In the eighteenth century Louis Pasteur and Hermann Müller-Thurgau recognized the bacterial causes of malolactic fermentation (MLF), but only in the middle of 1960s the responsible organism was isolated, characterised and firstly named as *Leuconostoc gracile* or *Leuconostoc citrovorum* (Pilone and Kunkee, 1965) and later as *Leuconostoc oenos* (Garvie, 1967). In 1995 it was reclassified as *O. oeni* by Dicks *et al.* (1995). This species shares a fairly small

DNA homology with the other genera in the *Leuconostoc* branch of the LAB (Dicks *et al.*, 1995).

Strains of *O. oeni* are described as ellipsoidal to spherical cells that usually occur in pairs or chains (Garvie, 1967; 1986; Holzapfel and Schillinger, 1992; Dicks *et al.*, 1995). Cells can be difficult to differentiate microscopically from short rods of *Lactobacillus* spp. The species is heterofermentative, converting glucose to equimolar amounts of D-lactic acid, CO_2, and ethanol or acetate (Krieger *et al*., 1993; Cogan and Jordan, 1994; Cocaign-Bousquet *et al.*, 1996). The bacterium hydrolyzes esculin and could produce ammonia from arginine (Pilone *et al*., 1991; Holzapfel and Schillinger, 1992; Dicks *et al.*, 1995). Most strains of *O. oeni* utilize L-arabinose, fructose, and ribose but not galactose, lactose, maltose, melezitose, raffinose, or xylose. Davis *et al*. (1988) determined that only 55% of the studied strains fermented ribose, 27% fermented D-arabinose, and 45% fermented sucrose.

O. oeni is well adapted to high ethanol concentrations (<15% v/v), low pH (as low as 2.9) and limited nutrient availability. These properties, associated with the ability to carry out the malolactic conversion and concomitant improvement of the wine sensory properties, has encouraged winemakers to control MLF by using selected LAB. However, despite this hardy nature it can be very unpredictable in the winery, and when it fails to perform as required it can incur great costs for the winemakers (Kunkee, 1991; Bartowsky, 2005).

1.2.2 Identification

Phenotypic methods have been widely used for wine LAB identification. However these techniques are tedious and very time consuming and the results could be imprecise and ambiguous. In the last decades, several molecular biology methods had become available wich allowed important advancements in the classification and identification of wine LAB.

The 16S rRNA gene sequence has been used to estimate phylogenetic relationships among LAB and also for the identification of unknown bacteria.

The identification is based on the similarity with other sequences within a database (Rodas *et al.*, 2003; du Plessis *et al.*, 2004). Acid nucleic hybridization (DNA-DNA and DNA-rRNA) methods have been successfully employed in the detection and identification of several wine LAB species in complex mixtures (Lonvaud-Funel *et al.*, 1989; 1991a, b; Sohier and Lonvaud-Funel, 1998). Some researchers had designed species-specific PCR methodologies to detect and identify grape juice and wine LAB (Zapparoli *et al.*, 1998; Bartowsky and Henschke, 1999; Guarneri *et al.*, 2001; Gindreau *et al.*, 2001; Spano *et al.*, 2002; Rodríguez *et al.*, 2007). Denaturing and temperature gradient gel electrophoresis (DGGE and TGGE) had been developed to quickly evaluate microbial communities by sequence-specific separation of PCR amplified fragments (16S rRNA and rpoB genes) (Cocolin *et al.*, 2001; Ercolini *et al.*, 2004; Bae *et al.*, 2006; Renouf *et al.*, 2006, 2007; Spano *et al.*, 2007). Real-time PCR (Furet *et al.*, 2004) also have been used for LAB species identification (Pinzani *et al.*, 2004). Randomly amplified polymorphic DNA (RAPD)-PCR has been used as a useful tool for identification and typing of wine LAB (Du Plessis and Dicks, 1995; Zavaleta *et al.*, 1997; Sohier *et al.*, 1999; Bartowsky *et al.*, 2003; Rodas *et al.*, 2005; Ruíz *et al.*, 2008). Restriction analysis of amplified DNA (ARDRA) has been used in the identification of lactobacilli (Rodas *et al.*, 2005) and *O. oeni* (Marques *et al.*, 2010). Pulsed-field gel electrophoresis (PFGE) revealed to be an efficient method for the differentiation of *O. oeni* strains (Pardo *et al.*, 1998; Zavaleta *et al*., 1997; Zapparolli *et al*., 2000; Sato *et al*., 2001; Ruíz *et al*., 2008). Multilocus sequence typing (MLST) was used by De la Rivas *et al*. (2004) and Bilhère *et al.* (2009) in order to discriminate *O. oeni* at the strain level. Lechiancole *et al.* (2006) used the molecular technique differential display PCR (DD-PCR) analysis for the differentiation of LAB species.

Table 3 summarized the chemotaxonomic and molecular methods used on the identification and differentiation of wine LAB.

Table 3 – Summary of the chemotaxonomic and molecular methods used on the identification of wine LAB.

Methods/techniques	Some References
Chemotaxonomic	
Fatty acids methyl esters	Kroppenstedt, 1985; Sasser, 1990; Guerrini *et al.*, 2003; Rodas *et al.*, 2005; Koort, 2006
Whole-cell proteins	Jackman, 1985; Couto and Hogg, 1994; Patarata *et al.*, 1994; Dicks *et al.*, 1995; Tenreiro, 1995; Ribéreau-Gayon *et al.*, 2006
Electrophoretic mobilities of enzymes	Garvie, 1986; Van Vuuren and Dicks, 1993; Dicks et al., 1995; Tenreiro, 1995; Sato *et al.*, 2001
Whole-cell composition	Van Vuuren and Dicks, 1993; Dellaglio *et al.*, 1994
Molecular	
DNA base composition	Priest and Austin, 1993; Van Vuuren and Dicks, 1993; Rosselló-Mora and Amann, 2001
DNA-DNA hybridization	Dicks *et al.*, 1995; Rosselló-Mora and Amann, 2001; Endo and Okada, 2006
16S rRNA gene sequencing	Vandamme *et al.*, 1996
Multilocus Sequence Typing (MLST)	De la Rivas *et al.*, 2004; Bilhère *et al.*, 2009
Pulsed-field gel electrophoresis (PFGE)	Kelly *et al.*, 1993; Tenreiro *et al.*, 1994; Tenreiro, 1995; Olive and Bean, 1999; Zavaleta *et al.*, 1997; Zapparoli *et al.*, 2000; Chambel, 2001; Sato *et al.*, 2001; Guerrini *et al.*, 2003; Larisika *et al.*, 2008
Restriction fragment length polymorphism (RFLP)	Tenreiro, 1995; Sato *et al.*, 2001 ; Rodas *et al.*, 2003, 2005 ; Rodríguez *et al.*, 2007
Amplified ribosomal DNA restriction analysis (ARDRA)	Rodas *et al.*, 2003; Marques *et al.*, 2010
Ribotyping	Tenreiro *et al.*, 1994 ; Tenreiro, 1995; Viti *et al.*, 1996
Randomly amplified polymorphic DNA (RAPD)-PCR	Zavaleta *et al.*, 1997; Zapparolli *et al.*, 2000 ; Reguant and Bourdon, 2003; Li *et al.*, 2006
Differential display PCR (DD-PCR)	Lechiancole *et al.*, 2006

1.2.3 Ecology

Prevailing LAB population in grape must includes strains of *Lactobacillus plantarum, L. casei, Leuconostoc mesenteroides*, *Pediococcus parvulus, P. pentosaceus* and *Oenococcus oeni* (Krieger, 2005). LAB can be found on grapes at low numbers (less than 10^3 CFU/g) (Lafon-Lafourcade *et al.*, 1983; Wibowo *et al*., 1985; Krieger, 2005). However acetic acid bacteria and yeasts are found in much higher numbers. Several studies showed that *O. oeni* is the predominant species carrying out MLF in wine, even though the composition of grape must at the beginning of the alcoholic fermentation (AF) is dominated by *Lactobacillus* spp. strains. *Pediococcus* spp. can be found mostly after MLF mainly in wines of higher pH. Wines of pH below 3.5 generally contain only *O. oeni*, while wine with pH above 3.5 can contain various species of *Pediococcus* spp. and heterofermentative strains of *Lactobacillus* spp. (Costello *et al*., 1985, Krieger, 2005). Wibowo *et al*. (1985) identified several stages of vinification where different species of LAB may occur and grow. Musts, soon after crushing, generally contain LAB at a population of 10^3 to 10^4 CFU/ml. The major species present at this stage include *L*. *plantarum* and *L*. *casei* and to a lesser extent *O. oeni* and *P*. *damnosus*. Most of these species normally do not multiply and die off during AF, although on unusual occasions (high pH wines) a slight proliferation of some mostly undesired ones may occur such as *Pediococcus* spp. and *Lactobacillus* spp. which subsequently spoil the wine due to the metabolism of subtractes existing in wine (sugars, tartaric acid and glycerol) (Costello *et al*., 1985). Sensitivity to ethanol may explain this decline in cell viability. After a lag phase, the length of which depends strongly on wine properties, the surviving cells start multiplying and after reaching the critical biomass (10^6 CFU/ml) degradation of malic acid initiates. Survival of the malolactic bacteria after completion of MLF strongly depends on wine conditions (namely ethanol content and pH) and on how the wine is handled. Addition of sulphur dioxide (SO_2) leads to a progressive loss of viability of these bacteria although the

wine pH is very important as it conditions the concentration of molecular SO_2 available, the fraction most active concerning antimicrobial effect. At low pH, wine LAB die off progressively, whereas at pH above 3.5 the LAB population may continue to increase (Krieger, 2005). Not only *O. oeni* but also spoilage bacteria develop levels as high as 10^6 to 10^8 CFU/ml and subsequently spoil the wine (Costello *et al*., 1985). Therefore, after MLF accomplishment, specifically at high pH conditions, early stabilization of the wine is recommended (Krieger, 2005).

1.2.4 Factors influencing LAB growth and survival in wine

The presence, growth and survival of LAB in wine could be influenced by chemical and physical composition of wine, technological factors and microbial interactions between the LAB and other wine microorganisms. All these factors vary according to the genus, species and LAB strain (Firme *et al*., 1994).

Wine is a complex environment and its physical and chemical characteristics diverge according to vine variety, climatic conditions and winemaking conditions. The principal physical and chemical factors are pH, ethanol content, SO_2 concentration and temperature (Versari *et al*., 1999).

pH is one of the most important parameters affecting the performance of LAB in wine. Generally, wines below pH 3.5 do not support the growth of *Pediococcus* and *Lactobacillus* spp., and invariably, *O. oeni* dominates in these wines. The incidence of *Pediococcus* and *Lactobacillus* spp. in wines increases as the pH approaches 4.0 (Wibowo *et al.*, 1985; Davis *et al.*, 1988; Ribéreau-Gayon *et al*., 2006).

Ethanol, a major metabolite resulting from the AF also affects the growth parameters of LAB and malolactic activity. The survivability and growth of LAB decreases as the ethanol concentration increases (Wibowo *et al.*, 1985). The cell membrane is also considered as the first target of ethanol (Garbay and Lonvoud-Funel, 1996; Teixeira *et al*., 2002). Ethanol presence induces an

increase in *O. oeni* membrane fluidity (Silveira *et al*., 2002, 2003; Chu-Ky *et al*., 2005). The composition of cell membrane is equally dependent on ethanol presence (Teixeira *et al*., 2002). Cells of *O. oeni* modify composition of fatty acids and its membrane during culture in the presence of ethanol. The proportion of cyclic fatty acids and the membrane protein/phospolipid ratio increases to limit the effect of ethanol on lipids (Teixeira *et al*., 2002; Silveira *et al*., 2003). Exposure to ethanol can equally induce a dissipation of the membrane electrochemical gradient (Silveira *et al*., 2002, 2004). An influx of protons can then occur which will affect cell processes dependant on the pH gradient such as ATP synthesis, transportation of amino acids and L-malate (Salema *et al*., 1996b). Ethanol concentration above 12% v/v inhibited malolactic activity (Capucho and San Romão, 1994).

The ideal temperature for growth of *O. oeni* in wine and consumption of L-malic acid is between 20 and 25ºC (Britz and Tracey, 1990). It is established that the optimum growth temperature rises as a consequence of the ethanol amount increasing. The normal temperature at which MLF is carried out in the cellars is between 18 and 22ºC. These conditions are therefore favourable for the growth of *O. oeni*. Nevertheless, in some cases temperature is often less than 18ºC, bacterial growth is then slower, enzymatic activities are reduced and MLF starts late (Wibowo *et al.*, 1985, Davis *et al.*, 1988, Britz and Tracey, 1990; Ribéreau-Gayon *et al*., 2006).

Sulphur dioxide is extensively used in winemaking as an antioxidant (protects wine from an excessively intense oxidation of phenolic compounds and certain elements of aroma), antiseptic (control/inhibits the growth of wild yeast and bacteria) and antioxidasic (inhibits the functioning of oxidation enzymes like tyrosinase and laccase and can ensure their destruction over time) (Fugelsang, 1997; Carreté *et al.*, 2002; Ribéreau-Gayon *et al.*, 2006). Normally, LAB have difficulty in developing at concentrations upper than 100 mg/L of total SO_2 and 10 mg/L of free SO_2 (Reguant *et al*., 2005). For a given strain, the sensitivity varies according to environmental growth

conditions and physiological adaptation possibilities (Britz and Tracey, 1990). Lafon-Lafourcade and Peynaud (1974) observed that cocci are less resistant to SO_2 presence than lactobacilli. *P. damnosus* ropy strains are insensitive to SO_2 doses that inhibit or kill other strains (Lonvaud-Funel and Joyeux, 1988).

Technological factors, as clarification of must and wine by sedimentation, filtration or centrifugation can remove a large proportion of LAB, reduce the possibility of MLF by indigenous flora and reduce the incidence of bacterial growth and its effect on wine quality. Moreover, these operations, if excessive, could also remove essential nutrients for the growth of LAB (Wibowo *et al.*, 1985). Wine fermented in contact with grape skins undergoes more consistent and rapid MLF than wine fermented in the absence of skins. Delayed racking or leaving the wine in contact with the lees after AF also stimulates MLF. This effect is apparently related to the leaching of stimulating substances from grape skins and nutrients provided by yeasts autolysis that promote LAB growth (Wibowo *et al.*, 1985). Other vinification factors that could influence the survival and growth of LAB include cold stabilization, ion exchange and heat pasteurization. Ion exchange and cold stabilization could remove the bacteria or important nutrients for their growth. The heat sensitivity of wine LAB varies with the species and wine properties. *O. oeni* is slightly more resistant to heat destruction than species of *Lactobacillus* and *Pediococcus* (Wibowo *et al*., 1995).

During the winemaking process, there is large microbial diversity. Mixed cultures of microorganisms establish the opportunity of antagonistic and synergistic relationships, but, in some cases, may have no effect. In winemaking, there is the possibility of the interaction of LAB with yeast, fungi, acetic acid bacteria and bacteriophages, as well as interactions between species and strains of LAB (Alexandre *et al*., 2004; Comitini *et al*., 2005).

The antagonistic effect of yeast has been explained by the competition for nutrients and the production of substrates that inhibit bacterial growth, such as ethanol or SO_2 or medium chain fatty acids. On the other hand, yeast may

support the growth of LAB. During extended lees contact with wine, the process of yeast autolysis releases vitamins and amino acids into the wine, therefore inducing wine nutrient enrichment (Alexandre *et al*., 2004; Comitini *et al*., 2005).

The competition also exists between different types of LAB. During the winemaking process, opposing effects between different LAB types can be found (Lonvaud-Funel and Joyeux, 1993). These effects are probably due to the production of components with antimicrobial properties such as bacteriocines (Yurdugül and Bozoglu, 2002).

O. oeni strains can be infected by phages (Henick-Kling *et al*., 1986). This phage attacks appear in wine together with a slowdown of MLF (Henick-Kling, 1995). The phages, isolated from wine, are able to induce lytic and lysogenic cycles in *O. oeni* (Henick-kling *et al*., 1986; Poblet-Icart *et al*., 1998). Sensitivity of *O. oeni* strains to phages is very variable. Difficulties in MLF due to phage can therefore result in a delay, thus allowing the development of undesirable bacteria such as *Pediococcus*. In certain cases, phage attacks can lead to a total MLF inhibition. Nevertheless, these attacks are limited by wine acidity (Henick-kling *et al*., 1986).

1.3 OENOLOGICAL IMPORTANCE OF LACTIC ACID BACTERIA

During the manufacture of red wines, after the AF, the MLF takes place by the action of LAB. Its main purpose is to reduce wine acidity, transforming malic acid into lactic acid. Moreover, during this process volatile compounds are formed, which enrich the wine aromatic quality (Henick-Kling, 1995; Revel *et al.*, 1999). On the other hand, during MLF there is the risk of some LAB produce undesirable compounds (off-flavour compounds, biogenic amines, ethyl carbamate, etc.) that can depreciate the wine (Bartowsky, 2005). The overall impacts of LAB on winemaking process are summarised in Figure 1.

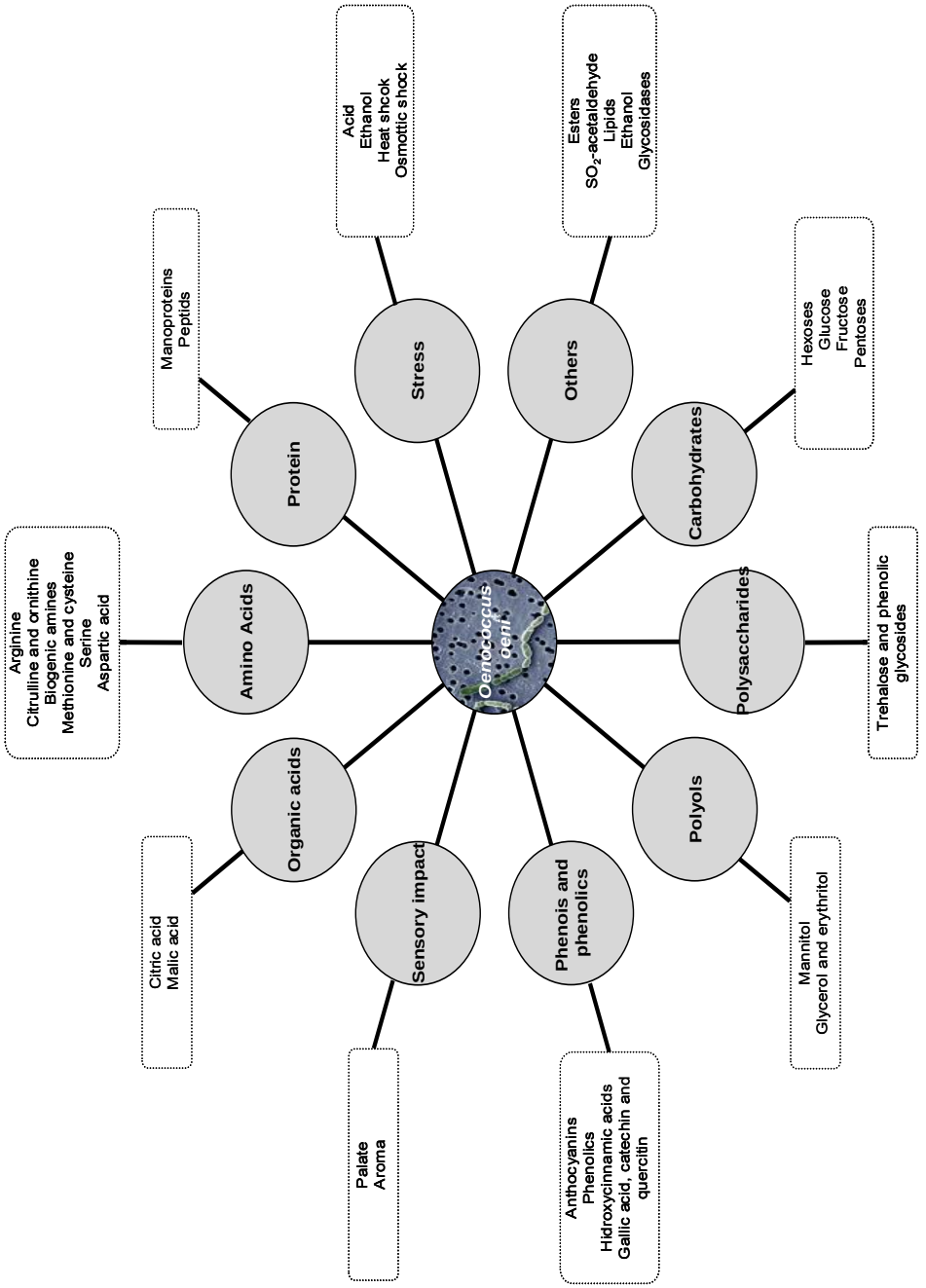

Figure 1 – Overview of the characterised biochemical changes which arise during MLF and *Oenococcus oeni* metabolism (adapted from Bartowsky, 2005).

1.3.1 Malolactic fermentation

MLF is a secondary fermentation that usually occurs at the end of AF, although sometimes can occur earlier. MLF results from the metabolism of malic acid by certain lactic acid bacteria, especially *O. oeni* (Dicks *et al.*, 1995). This process consists in the conversion of the dicarboxylic L-malic acid into monocarboxylic L-lactic acid and carbon dioxide by the malolactic enzyme (IUC number 1.1.1.38) in the presence of NAD^+ and Mn^{2+} (Henick-Kling, 1993; Salema *et al*., 1996a, b; Versari *et al.*, 1999; Liu, 2002) which result in an increase in pH and a decrease in titratable acidity (Davis *et al*., 1985; Liu, 2002; Bartowsky, 2005). LAB carried out the MLF during the vinification of most red and certain white and sparkling wines (Davis *et al.*, 1988; Kunkee, 1991; Lonvaud-Funel, 2001; Bartowsky *et al.*, 2002).

In the last decades, several authors dedicated their efforts studying the benefits of the malolactic conversion to the bacterial cell. Since no substrate level phosphorylation (and ATP production) is directly linked to the decarboxylation of L-malic acid by the malolactic enzyme, it has been difficult to understand the physiological advantage of the MLF for the bacterial cell.

In 1976, Pilone and Kunkee demonstrated that, during MLF by *O. oeni*, the rate of growth at low pH was increased, independently of the change of pH in the growth medium.

Cox and Henick-Kling (1989) showed that ATP was produced during MLF and that its production was linked to proton motive force (Δp) across the cell membrane and functional ATPase. This general mechanism apparently works in all LAB which possess the malolactic enzyme (Cox and Henick-Kling, 1990). The ATP production from malolactic fermentation is independent of glucose metabolism (Cox, 1991). In the model presented by Olsen *et al.* (1991), one molecule of malate enters the cell, is decarboxylated, and one molecule of lactate leaves the cell with one H^+; this is equal to the translocation of one H^+ to the outside (Figure 2) (Cox and Henick-Kling, 1995).

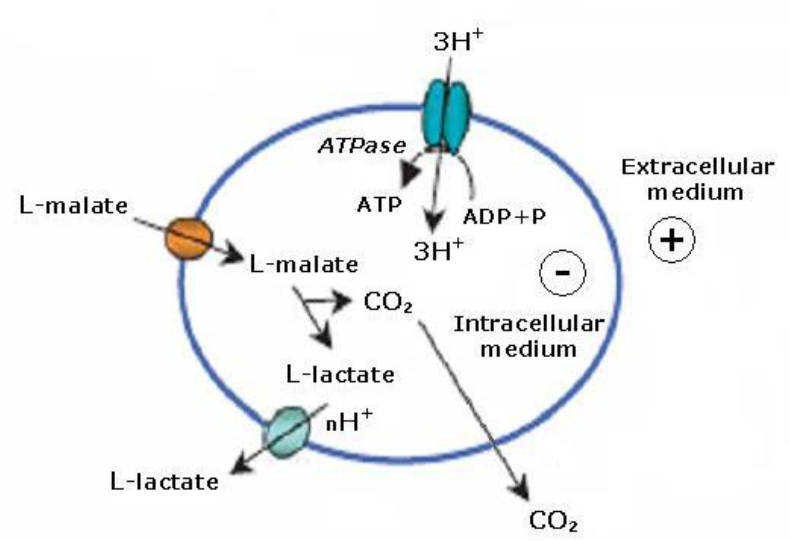

Figure 2 – Model of the ATP-generating mechanism for the malolactic conversion (adapted from Bartowsky, 2005).

The malolactic conversion is a pathway linked to the proton motive force for the generation of ATP during growth at low pH. The system does not provide carbon for growth; therefore the bacteria must also be able to utilize sugars as a carbon source. In LAB isolated from wine, malate apparently is preferred as an energy source during growth at low pH and at the beginning of cell growth. Malate-dependent ATP synthesis can be initiated in starved cells. The increased intracellular pH, Δp and ATP from MLF help the cell to begin its growth. Also, the increased extracellular pH allows extended growth (Cox and Henick-Kling, 1989, 1990; Kunkee, 1991; Henick-Kling, 1995).

Based on studies of intracellular pH and Δp in cells during MLF, it was proposed that *O. oeni* obtained an energetic advantage from an increased intracellular pH and from an increased Δp (Salema *et al.*, 1996a, b).

The genes involved in the malolactic reaction are *mle*A that encodes the malolactic enzyme, *mle*P that encodes the malate permease and *mle*R (malolactic regulator) that is a regulatory protein. Those genes were cloned

and their respective proteins characterised (Labarre *et al*., 1996a, b). The genes are present in a cluster with the *mle*A and *mle*P in a single operon, and *mle*R transcribed in the opposite direction (Figure 3). The regulatory protein is a typical DNA binding protein with the classical helix-turn-helix motif, and belongs to the LysR-type regulatory protein (Schell, 1993). With a better understanding of the regulation of *mle*A expression during MLF, more efficient induction of MLF might be possible (Galland *et al*., 2003; Bartowsky, 2005).

Figure 3 – Genetic organization of the malolactic operon *mle*RAP (adapted from Bartowsky, 2005).

1.3.2 Glycosidic enzymes

O. oeni is the species generally recognized as beneficial for final wine aroma (Henick-Klling, 1995; Maicas *et al*., 1999). During MLF, LAB modify the flavour profile of wine with a broad range of secondary compositional changes that include amino acid metabolism, metabolism of polyols, ester synthesis and hydrolysis of glycosides (Liu, 2002).

The use of enzyme preparations, such as pectinases, to improve wine characteristics is a common practice in oenology. An increased interest has been focused on glycosidic enzymes, in particular β-D-glucopyranosidase (EC 3.2.1.21), as a means of flavour modification of wine. These enzymes hydrolyse glycoconjugated precursors, releasing active aroma compounds and free phenols (Spagna *et al*., 2000; Barbagallo *et al*., 2002).

Several studies have focused on LAB glycosidase activities that can release compounds with potential sensory significance (Boido *et al.*, 2002; Mansfield *et al.*, 2002; D'Incecco *et al.*, 2004). Release of glycosylated volatile precursors in Tannat wine was also observed (Boido *et al*., 2002), and only minimal *O. oeni* glycosidase activity was noted in Viognier glycosidic extracts (McMahon *et al*., 1999). The effective β-glucosidase activity of some strains of *O. oeni* has been however reported (Grimaldi *et al*., 2000; Boido *et al*., 2002; Barbagallo *et al*., 2004; D'Incecco *et al*. 2004). Mansfield *et al*. (2002) had also detected the production of β-glucosidase enzymes in several strains of *O. oeni*, although cultures of the same strains failed to hydrolyse native grape glycosides.

Specific strains of LAB, well-adapted to perform MLF, might represent a source for β-glucosidase enzymes capable to operate under the physicochemical conditions of wine, hence influencing its flavour complexity. The use of such strains with that purpose might be advantageous with respect to yeasts: LAB are generally inoculated after AF directly into wine, this being a more stable system than must; in addition, LAB show less undesirable enzyme activities, which might interact with some components of wine, thus affecting its quality. All these factors can increase the specificity towards glycosidic components (Grimaldi *et al*., 2000; Boido *et al*., 2002; Mansfield *et al*., 2002). Products of grape glycoside hydrolysis include monoterpenes, norisoprenoids, C6 alcohols and benzene derivatives. Glycosides can be either monoglycosides or dissacharide glycosides in which the glucose bound to the aglycon moiety is substituted by an α-L-arabinofuranoside, β-D-rhamnopyranoside, or β-D-xylopyranoside. The release of aglycons from disaccharides involves the sequential liberation of the sugars with specific glycosidases (Günata *et al.*, 1988). It has been demonstrated that *O. oeni* was able to cleave the glucose moiety from the major red wine anthocyanin, malvidin-3-glucoside, to use it as a carbon source.

The study of the hydrolysis of wine aroma precursors (linalool, α-terpineol, nerol and geraniol) during MLF has been carried out with some *O. oeni* starter cultures in model wine solutions by Ugliano *et al*. (2003). The release of glycosidically bound aroma compounds was evaluated for diverse strains that also carried out the MLF. Although the amount of released precursors was strain dependent (Grimaldi *et al*., 2000; Boido *et al*., 2002; Mansfield *et al*., 2002).The large release of glycosylated aroma compounds observed during their experiments suggests that *O. oeni* can actively contribute to the changes of sensory characteristics of wine after MLF through the hydrolysis of aroma precursors (Ugliano *et al*., 2003).

Food safety is a goal which importance has been increasing over the last decades. Apart from the primary metabolic products and many flavour compounds (both desirable and undesirable) released during the fermentations, some microrganisms produce secondary metabolic products that may affect the wholesomeness of the fermented foods (dairy products, beer, wine, cider, fermented sausages, etc.). Biogenic amines (BA) and ethyl carbamate (EC) (Lonvaud-Funel and Joyeaux, 1994; Silla Santos, 1996) are two of such group of compounds.

Once the winemaking process is well controlled, the multiplication and subsequent biochemical reactions supported by LAB improve wine quality and stability. However, they may also make it unmarketable under certain conditions. A wine fault is an unpleasant characteristic that result from poor winemaking practices or storage conditions sometimes leading to wine spoilage. Many of the compounds that cause wine faults are already naturally present in wine but at low concentrations. When the concentration of these compounds greatly exceeds the sensory threshold, they obscure the positive flavours and aromas of wine. Eventually the quality of the wine is reduced, making it less appealing and sometimes undrinkable.

1.3.3 Biogenic amines

Amines have an important metabolic role in living cells. Polyamines are essential for growth. (Silla-Santos, 1996).

Biogenic amines are organic nitrogenous bases of low molecular weight, with a biological activity, which may be formed and degraded during the normal metabolism of animals, plants and microorganisms (Ten Brink *et al.*, 1990). BA are sources of nitrogen and precursors for the synthesis of hormones, alkaloids, nucleic acids, and proteins (Silla-Santos, 1996). These compounds can have an aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine) structure. Putrescine, spermine and spermidine are present in plants, where they are important for physiological processes such as flowering and fruit development, cell division, stress responses and senescence (Halász *et al.,* 1994).

BA are widely present in foods and beverages that contain proteins or free amino acids. Such foodstuffs include fish, meat products, eggs, cheeses, nuts, fermented and fresh fruits and vegetables, beers and wines (Halász *et al*., 1994; Silla-Santos, 1996).

In fermented foods, BA are a consequence of the decarboxylation of their respective free precursor amino acids (Figure 4) through the action of substrate-specific microbial decarboxylases (ten Brink *et al*., 1990; Halász *et al*., 1994).

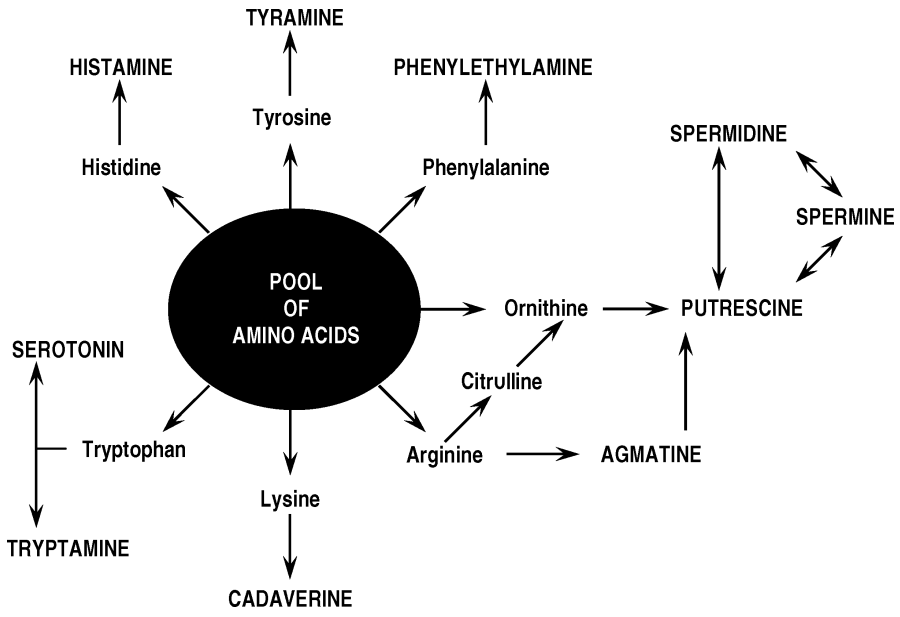

Figure 4 – Precursor amino acids of biogenic amines (adapted from Ancín-Azpilicueta *et al*., 2008).

The presence of BA in food products can be undesirable because if absorbed at a too high concentration, they may induce headaches, respiratory distress, heart palpitation, hyper or hypotension, and several allergenic disorders. The consumption of wine containing high concentrations of BA is claimed to induce toxicological effects in sensitive humans (Silla-Santos, 1996).

Generally, if a low concentration of BA is ingested, they are quickly detoxified in the human body by amine oxidases or through conjugation. Amine oxidases catalyse the oxidative deamination of BA to produce an aldehyde, hydrogen peroxide and ammonia (Gardini *et al*., 2005). However, if an excessive amount of BA is ingested or if the normal catabolic routes are inhibited or genetically deficient, some physiological disorders can occur (ten Brink *et al.*, 1990). The possible toxic effect of BA depends on the tolerance of the individual for the compound, concentration of total BA and consumption of

ethanol and/or drugs. Individuals suffering from histamine intolerance, due to a reduced activity of amino-oxidase (MAO) enzymes, and individuals under antidepressive treatment with monoamino-oxidase inhibitor (MAOI) drugs are particularly sensitive to these biologically active amines. In addition, MAO are competitively inhibited by other biogenic diamines (putrescine and cadaverine) and alcohol, which can enhance the toxicity of aromatic biogenic amines in wine (Wantke *et al*., 1993).

Kanny *et al*. (2001) reported that a normal individual can tolerate 120 mg/L of histamine taken orally before symptoms occur, but only 7 µg administered intravenously. However, other studies conclude that no relationship exists between the oral ingestion of BA and wine intolerance. Jansen *et al*. (2003) found no relation between the oral ingestion of BA and food intolerance reactions. There is therefore no scientific basis for dietary recommendations concerning BA in such patients.

Currently, a lack in legislation about the permitted levels of amines in wine affects the import and export of wine (Ancín-Azpilicueta *et al.*, 2008). However, some countries have fixed upper limits for histamine (10 mg/l in Switzerland and Austria; 3 mg/l in Netherlands; 2 mg/l in Germany; 5 mg/l in Finland; 8 mg/l in France and 5 to 6 mg/l in Belgium) (Bauza *et al.*, 1995).

1.3.3.1 Methods for the detection of biogenic amines

During the last decades, several methods have been developed for the detection of BA. Some detection methods are based on differential growth media signalling the increase of the pH upon BA formation (Niven *et al.*, 1981; Choudhury *et al.*, 1990; Maijala, 1993; Bover-Cid and Holzapfel, 1999; Mavromatis and Quantick, 2002), enzymatic methods for the determination of BA with transglutaminase (Punakivi *et al.*, 2006) and specific enzymatic methods for histamine-producing bacteria based in the production of hydrogen peroxide by an oxidase enzyme action on histamine (Landete *et al.*, 2004). Several analytical methods have been described and recommended for

identification and quantification of BA in food and beverage so far, including thin-layer chromatography (TLC) (Halász *et al.*, 1994; Gárcia-Moruno *et al.*, 2005), high-performance liquid chromatography (HPLC) (Veciana-Nogues *et al.*, 1995; Hernández-Jover *et al.*, 1996; Romero *et al.*, 2002; Vidal-Carou et al., 2003; Landete *et al.*, 2005a; Gómez-Alonso *et al.*, 2007), gas chromatography (GC) (Fernandes and Ferreira, 2000) and capillary electrophoresis (CE) (Kovács *et al*., 1999). HPLC is the method routinely used for BA analysis that involves an extraction with methanol, subsequent ion-exchange chromatography, and a pre-or-post column derivatization step. Several derivatization reagents have been tested. Generally, for a pre-column derivatization is used the dansyl chloride and for post-column derivatization o-phthalaldehyde (OPA) is used (Karovičová and Kohajdová, 2005).

Several studies describing LAB loss of ability to produce BA after prolonged storage or cultivation of isolated strains in synthetic media have been reported (Lonvaud-Funel and Joyeux, 1994; Izquierdo-Pulido *et al.*, 1997). Therefore, during the last decade several molecular methods have been described for the early detection of BA-producing bacteria which become an alternative to traditional culture methods. PCR and DNA hybridization have become important methods and offer the advantages of speediness, reliability, culture-independent, sensitivity, simplicity and specific detection of targeted genes that encode for the decarboxylase enzymes (Marcobal *et al.*, 2006 and Landete *et al.*, 2007a, b). Since an intrinsic disadvantage of PCR is the detection of non-viable cells, the ability to distinguish between viable and non-viable organisms is crucial when PCR is used for risk assessment of BA detection.

1.3.3.2 Biogenic amines in Portuguese wines

During the last decades some scientific work were published about the presence of BA in Portuguese wines (Mafra *et al*., 1999; Herbert *et al*., 2005; Leitão *el at*., 2005).

Mafra *et al*. (1999) shown that the BA (histamine, tyramine and phenyletylamine) suspected to cause toxicological effects do not represent any concern in the wines tested (30 Portuguese wines, including fortified wines: Porto, Madeira and Moscatel de Setúbal; red D.O.C. wine Dão and white D.O.C. wine Vinho Verde) as their amounts generally do not exceed in general 5 mg/l. Concentrations of BA (putrescine and cadaverine) associated with deficient sanitary conditions the contents are also very low ranging between 0.2 and 6 mg/l. Tryptamine was not detected in any wines.

Herbert *et al*. (2005) analysed a large number of samples (209) comprising wines and musts from different varieties, Alentejo sub-regions and vintages. For the majority of the samples, the amines cadaverine, tryptamine, β-phenylethylamine and isoamylamine were found to be below the limit of detection of the method used. No significant increase in the levels of total volatile amines was observed during AF or spontaneous MLF. While higher histamine levels were only found during the storage period, an increase in the concentration of tyramine was confirmed in red wines immediately after MLF, which seems to be also the main origin of putrescine.

Leitão *et al*. (2005) evaluate the types and levels of BA in commercial Portuguese wines from different regions and over a period of 10 years. This work showed that most of the commercial Portuguese wines presented a low concentration of BA (< 8 mg/l) and 25% of these wines presented contents lower than 2.5 mg/l. Tyramine and putrescine were the BA present in a higher concentration. The wines from the Alentejo region showed highest concentrations of BA, especially in tyramine and histamine contents. The concentrations of the BA in white wines never exceeded 17 mg/l, although in red wines they could reach 28 mg/l. According to the results, the authors could

not establish an effective correlation between some external factors directly associated with the technological process of wine production (soil, pH, and ethanol content) and the detected levels of BA.

1.3.3.3 Formation of biogenic amines

In wine, BA are formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (ten Brink *et al*., 1990). The presence and accumulation of BA depend on many factors such as the presence of specific bacteria, availability of free amino acids, existence of appropriate environment, ripening and storage period and conditions. A great variability characterizes BA contents of wines produced in different geographical areas. It is dependent upon the pedoclimatic characteristics of the production area (kind of soil, nitrogen fertilizer and climacteric conditions) as well as winemaking conditions, such as grape variety, grape maturation degree, vinification method, duration of initial fermentation phase, oenological treatments (levels of sulphur dioxide addition), pH, the duration of grape skin maceration, growth of certain lactic acid bacteria and yeasts, content of precursor amino acid, and ageing of wine on lees (Zee *et al*., 1983; Vidal-Carou *et al.*, 1990, 1991; Lounvaud-Funel and Joyeux, 1994; Glória *et al.*, 1998; Coton *et al.*, 1999; Torrea-Goni and Ancín-Azpilicueta, 2002; Landete *et al*., 2005b; Martin-Alvarez *et al*., 2006; Ancín-Azpilicueta *et al*., 2008; Marques *et al*., 2008).

The physiological function of amino acid decarboxylative pathways in bacteria is still not entirely clear. It seems that for LAB the accumulation of amines is a mechanism of protection against the acidid media (Lonvaud-Funel, 2001; van de Guchte *et al*., 2002) and a way of obtaining metabolic energy through coupling amino acid decarboxylation with electrogenic amino acid/amine antiporters (Konnings *et al*., 1997). Furthermore, the production of polyamines such as putrescine can interfere in other physiological functions in bacteria

such as osmotic stress (Schiller *et al*., 2000) and oxidative stress responses (Tkachenko *et al*., 2001).

In the formation of BA in wine an important role is attributed to the LAB responsible for carrying out the MLF. In fact, several authors sugested that LAB are responsible for large accumulations of those compounds in wine (Bauza *et al*., 1995; Soufleros *et al*., 1998; Gerbaux and Monamy, 2000). Table 4 shows the LAB species capable of producing different BA. Marcobal *et al*. (2006) found a general increase in the concentration of amines in the first part of MLF. These results along with the studies made *in vitro* by Pessione *et al*. (2005) seem to indicate that the biosynthesis of the amino acid decarboxylase enzymes could take place at the exponential and at the end of the bacterial growth stage.

Table 4 – Species of LAB able to produce biogenic amines in wine (adapted from Ancín-Azpilicueta *et al*., 2008).

Biogenic amines	Species of lactic acid bacteria	Some References
Histamine	*Oenococcus oeni*	Lonvaud-Funel and Joyeux, 1994; Coton *et al*., 1998b; Guerrini *et al*., 2002; Landete *et al*., 2005b
	Lactobacillus hilgardii	Farías *et al*., 1993; Landete *et al*., 2005b; Lucas *et al*., 2005 ; Constantini *et al*., 2006
	Lactobacillus sp. 30a	Moreno-Arribas *et al*., 2003; Constantini *et al*., 2006
	Pediococcus damnosus	Aerny, 1985; Delfini, 1989
	Pediococcus parvulus	Landete *et al*., 2005b
Tyramine	*Lactobacillus brevis*	Moreno-Arribas *et al*., 2000; Lucas and Lonvaud-Funel, 2002; Lucas *et al*., 2003; Landete *et al*., 2007a, b; Constantini *et al*., 2006
	Lactobacillus hilgardii	Moreno-Arribas *et al*., 2000 ; Landete et al., 2007a, b
	Leuconostoc mesenteroides	Moreno-Arribas *et al*., 2000
Phenylethylamine	*Lactobacillus brevis*	Landete *et al*., 2007a, b
	Lactobacillus hilgardii	Landete et al., 2007a, b
Putrescine	*Oenococcus oeni*	Coton *et al*., 1999; Guerrini *et al*., 2002; Marcobal *et al*., 2004; Mangani *et al*, 2005
	Lactobacillus sp. 30a	Gale, 1946, Tabor and Tabor, 1985
	Lactobacillus hilgardii	Arena and Manca de Nandra, 2001
	Lactobacillus plantarum	Arena and Manca de Nandra, 2001
	Lactobacillus buchneri	Moreno-Arribas *et al*., 2003

Nevertheless, other authors have not found any relation between the presence of LAB in wine and the occurrence of BA. Buteau *et al.* (1984) have shown that BA, especially histamine, decreased during MLF. Ough *et al*. (1987) studied the capacity of different LAB (*Lactobacillus*, *Oenococcus* and *Pediococcus*) to produce histamine from histidine under different fermentation conditions and they did not find significant amounts of histamine from

decarboxylation of histidine either in model solutions or in fermented juice samples. This variability in the results could be explained by the fact that the LAB of wine have a different capacity for producing amines, and this capacity appears to be strain dependent and not related to species specific characteristics (Coton *et al*., 1998a, b; Bover-Cid and Holzapfel, 1999; Leitão *et al*., 2000; Landete *et al*., 2005a, b).

As a consequence of the LAB variability for producing BA, several studies have been carried out over the last years where several LAB strains, producers of BA, especially histamine, tyramine and putrescine, have been isolated and characterized at a biochemical and molecular level.

1.3.3.4 Histidine decarboxylase

The enzyme histidine decarboxylase (HDC) (EC 4.1.1.22) catalyzes the conversion of histidine present in wine into histamine and CO_2. There are two distinct classes of histidine decarboxylases: eukaryotic and gram-negative bacteria HDC require pyridoxal phosphate as a cofactor, whereas HDC of gram-positive bacteria use a covalently bound pyruvoyl moiety as a prosthetic group (Recsei *et al.*, 1983; Recsei and Snell, 1984). Histidine decarboxylase activity has been reported in many genera of γ -proteobacteria, clostridia, and LAB, although this ability appears to be strain-dependent (Halász *et al.*, 1994; Marino *et al.*, 2000). Within LAB, HDC has been detected in some strains of *Lactobacillus*, *Oenococcus*, *Pediococcus*, and *Tetragenococcus* (Rice and Koehler, 1976; Recsei *et al*., 1983; Lonvaud Funel and Joyeux, 1994; Satomi *et al.*, 1997; Lucas *et al.*, 2005).

The histidine decarboxylase gene (*hdc*A) encodes a single polypeptide of 315 amino acids. This gene has been identified in different gram-positive bacteria, such as *Clostridium perfringens* (van Poelje and Snell, 1990), *O. oeni* (Coton *et al.*, 1998b), *Lactobacillus buchneri* (Martín *et al.*, 2005), *Lactobacillus hilgardii* (Lucas *et al.*, 2005), *Tetragenococcus muriaticus* (GenBank acc. Nº AB040487) and *T. halophilus* (GenBank acc. Nº AB076394)

and also in different gram-negative bacteria, such as *Morganella morganii*, *Raoultella planticola*, *Enterobacter aerogenes*, *E. amnigenus*, *Photobacterium damselae*, *P. phosphoreum*, *Hafnia alvei*, *Erwinia* sp. and *Proteus vulgaris* (Takahashi *et al.*, 2003). In all of these bacteria, *hdcA* constitutes an operon together with a downstream gene (*hdc*B) of unknown function. In addition, the gene *hdc*P encoding the histidine/histamine antiporter is located upstream of the *hdc*AB operon whereas a gene encoding a histidyl-tRNA synthetase (*his*RS) is located downstream in *L. buchneri* (Martín *et al.*, 2005) and *L. hilgardii* (Lucas *et al.*, 2005) (Figure 5).

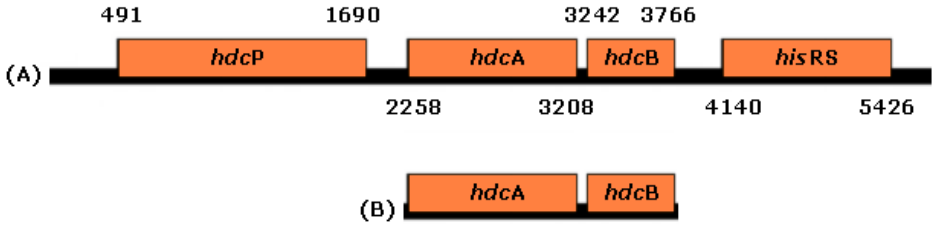

Figure 5 – Genetic organization of loci histidine decarboxylase of *Lactobacillus hilgardii* (A) and *Oenococcus oeni* (B) (adapted from Lucas *et al*., 2005).

The comparison of *hdc* clusters reveals a very high similarity both in amino acid sequence and gene organization. This, together with their irregular distribution and the localization of the *hdc* genes on a plasmid, at least in *L. hilgardii*, suggests that some strains have acquired these genes by horizontal gene transfer. Clearly, the plasmid harboring the *hdc* cluster in *L. hilgardii* IOEB 0006 was segregationally unstable (Lucas *et al.*, 2005), which might explain the low number of HDC positive strains isolated compared to the presence of histamine in fermented food and beverages. Transcriptional analyses of *hdc* clusters indicate that the expression of the *hdcAB* operon is

induced by histidine in *Lactobacillus* sp. 30a (Copeland *et al.*, 1989). In *L. buchneri*, *hdc*AB and *his*S are transcribed as a policistronic mRNA although both monocistronic and bicistronic mRNAs corresponding to *his*S and *hdc*AB, respectively, have been also detected. Histidine apparently induces the expression of *hdc*AB, although the mechanism of induction remains to be elucidated (Martín *et al.*, 2005).

Moreover, the comparison of the enzyme structures obtained under different pH conditions showed that the enzyme folds into the active form at acidic pH, whereas neutral and alkaline pH induce structural changes on the tertiary structure of the HDC monomers which disrupt the substrate binding site, thus greatly reducing its activity. Accordingly, HDC activity regulation depends on the internal pH (Schelp *et al.*, 2001). This regulatory effect of pH on the activity of HDC would ensure that the pathway is only active in acidic conditions and this agrees with the suggestion that His decarboxylation can act as a mechanism of protection against acidic pH (Fernández and Züñiga, 2006).

1.3.3.5 Tyrosine decarboxylase

Tyrosine decarboxylase (TYRDC) (EC 4.1.1.25) enzyme, accounting for tyramine formation, belongs to the group of pyridoxal phosphate (PLP)-dependent enzymes (Moreno-Arribas and Lonvaud-Funel, 1999, 2001). Only a few reports have described physiological studies of the influence of some physicochemical factors, such as temperature, pH, NaCl, or tyrosine concentration, on tyramine production by *Enterococcus faecalis*, *Lactobacillus curvatus*, *L. brevis and L. hilgardii* (Moreno-Arribas *et al.*, 1999; Connil *et al.*, 2002; Lucas *et al.*, 2003). Tyramine production seems to be not widespread among *O. oeni* strains (Moreno-Arribas *et al*., 2000, 2003; Geurrini *et al*., 2002). As far as literature repported until now, only two *O. oeni* strains showed ability to produce tyramine in a laboratory medium (Choudhury *et al.*, 1990; Gardini *et al*., 2005). TYRDC purification and characterization as well as the *tyr*DC gene sequencing have been reported only for *Enterococcus faecalis*

and *L. brevis*. Lucas *et al.* (2003) obtained the complete sequence of *tyrDC* operon (7979 bp). This operon comprises the tyrosyl-tRNA synthetase genes (*tyr*RS), tyrosine decarboxylase gene (*tyr*DC), amino acid permease gene (*tyr*P), Na^+/H^+ antiporter gene (*nha*C) and ornithine transcarbamylase genes (*otc*) (Figure 6).

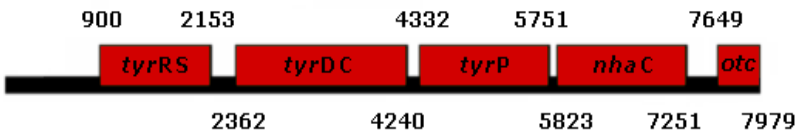

Figure 6 – Genetic organization of *tyrDC* operon of *Lactobacillus brevis* (adapted from Lucas *et al.,* 2003).

1.3.3.6 Ornithine decarboxylase

Ornithine decarboxylase (ODC, EC 4.1.1.17) is a PLP-dependent enzyme which catalyses the conversion of ornithine to putrescine. Several bacteria contain two forms of ODC: a biosynthetic or constitutive enzyme, expressed when bacteria are grown under neutral pH in minimal culture media, and a biodegradation or inducible form occuring under low pH conditions in rich media. This fact suggests that ODC can play a role in maintaining pH homeostasis. Among LAB, an inducible ODC, structured as a dodecamer of about 1000 kDa and requiring PLP as cofactor, has been described in *Lactobacillus* sp. 30a (Momany *et al*., 1995; Vitali *et al*., 1999). ODC from this LAB was sequenced (Hackert *et al.*, 1994) and its three-dimensional structure was also determined (Momany *et al.*, 1995). The ornithine decarboxylase gene from a putrescine-producer strain of *O. oeni* had also been sequenced (Figure 7) (Marcobal *et al.*, 2004).

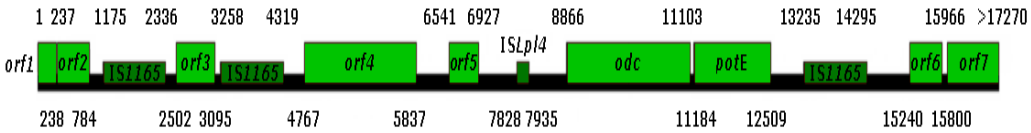

Figure 7 – Genetic organization of the 17.2 kb gene cluster of *Oenococcus oeni* RM83 (adapted from Marcobal *et al.*, 2004).

The origin of putrescine earns more detailed description because high amounts of this diamine in wine cannot be only dependent on the contents of free ornithine, since this amino acid concentration is low in musts and wine. In reality, ornithine may also be produced from the catabolism of arginine, one of the major amino acids found in grape juices, mostly metabolised by yeasts during AF, but generally still present in wines at significant levels before the onset of MLF. Arginine is also catabolized by several strains of LAB (lactobacilli and oenococci) via the arginine-deiminase pathway (Liu and Pilone, 1998).

Mangani *et al*. (2005) showed that some *O. oeni* strains demonstrated to have capability to produce putrescine only from ornithine, but other strains were able to produce putrescine also from arginine. In this study was also demonstrated that one *O. oeni* strain was capable to metabolize arginine to ornithine but was unable to decarboxylate ornithine to putrescine. Another strain was capable to produce putrescine from ornithine but unable to degrade arginine.

1.3.4 Arginine deiminase pathway

Two different pathways have been described for bacterial degradation of arginine: the arginine-urease pathway, which involves the enzyme arginase with the formation of ornithine and urea, and, more commonly, the arginine deiminase (ADI) (or dihidrolase) pathway.

The ADI pathway involves the following enzymes: arginine deiminase (ADI; EC 3.5.3.6), ornithine transcarbamylase (OTC; EC 2.1.3.3), and

carbamate kinase (CK; EC 2.7.2.2). Arginine is at first converted into citrulline, which is subsequently transformed into ornithine and carbamyl phosphate. This last compound provides ATP generation by using adenosine-phosphate (ADP). The degradation of 1 mol of arginine results in the formation of 1 mol of ATP and 2 mol of ammonia (Figure 8). ADI catalyzes the first step of the reaction, which degrades arginine to citrulline and ammonia. Citrulline is cleaved by OTC into ornithine and carbamyl-phosphate. OTC can also catalyze the reverse reaction, the synthesis of citrulline from ornithine and carbamyl-phosphate, a key step in the arginine biosynthetic pathway and the first step of the urea cycle. Nevertheless, OTCs are specialized in either a catabolic or an anabolic role and organisms that possess both pathways encode distinct OTCs. Finally, carbamyl-phosphate is utilized by carbamate kinase to phosphorylate ADP. The resulting carbamate spontaneously splits into ammonia and CO_2 (Mira de Orduña *et al*., 2000, 2001; Zúñiga *et al.*, 2002; Divol *et al*., 2003).

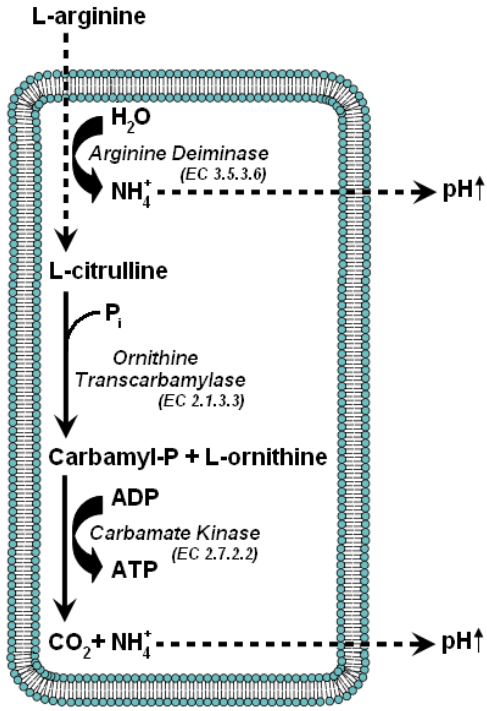

Figure 8 – Metabolic pathway of arginine by *Oenococcus oeni* (adapted from Mira de Orduña *et al.*, 2001).

The ADI pathway is widely distributed among LAB. It has been described in strains belonging to genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus*, and *Weissella*. Complete *arc* gene clusters associated with the ADI pathway, have been characterized in a number of LAB (Zúñiga *et al.*, 1998, 2002; Tonon *et al.*, 2001; Dong *et al.*, 2002; Barcelona-Andrés *et al.*, 2002; Divol *et al.*, 2003).

The complete sequencing of several LAB genomes has allowed the identification of some additional clusters (Arena *et al.*, 2002; Spano *et al.*, 2004). The reported results have evidenced that the organization of the ADI gene clusters is particularly complex in LAB when compared to other organisms. In addition to the structural genes, LAB *arc* clusters may include genes that encode transport proteins, a putative aminotransferase belonging

to the subfamily I (*arc*T), and regulatory genes belonging to ArgR and Crp/Fnr families. In some species, duplicated genes may also be present. The regulation of the ADI pathway differs among LAB, and these differences seem to correlate with their adaptation to different habitats. The ADI cluster of *O. oeni* includes the *arc*A (ADI), *arc*B (OTC), and *arc*C (CK), all of them with expression induced by arginine, and two duplicated *arc*D genes (*arc*D1 and *arc*D2), constitutively expressed and putatively coding for membrane proteins involved in arginine transport. The product of *arc*R, the sixth gene of the *arc* operon is essential for the arginine deiminase pathway expression (Figure 9) (Tonon *et al.*, 2001, Zúñiga *et al.*, 2002; Divol *et al.*, 2003).

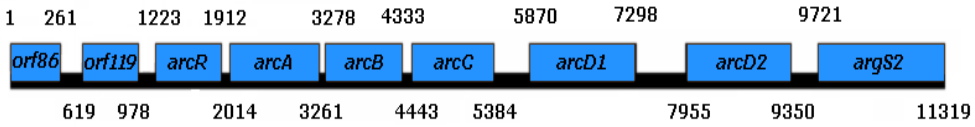

Figure 9 – Genetic organization of ADI gene cluster in *Oenococcus oeni* (adapted from Divol *et a*l., 2003 and Nehme *et a*l., 2006).

The transport of arginine in LAB is mediated by an antiporter that exchanges arginine and ornithine. The exchange is electroneutral, proton motive force independent and driven by a gradient of concentration of ornithine across the membrane (Driessen *et al.*, 1987). The antiporter appears to be the main arginine transport system associated with the ADI pathway in LAB. Even though, there is some evidence suggesting that other transporters may be involved in arginine transport in LAB (Fernández and Züñiga, 2006).
Arginine metabolism could originate two undesirable substances, ethyl carbamate and putrescine, which is a concern in wine industry.

1.3.2.3 Ethyl carbamate

Ethyl carbamate (EC) (also known as urethane), a carcinogen agent, has a range of biological activities including its ability to induce some kinds of tumours in animals and humans. This compound occurs naturally in fermented beverages and foods such as beer, wine, bread and yoghurt, but normally in low concentration (few μg/L) (Battaglia *et al*., 1990). Public health concern about EC in alcoholic beverages arose in 1985 when relatively high levels were detected by Canadian authorities (Conacher and Page, 1986). Consequently, Canada set levels of EC in beverages (30 μg/L for wines, 100 μg/L for fortified wines, 150 μg/L for wine spirits, brandies, and whiskies, and 400 μg/L for fruit brandies, cordials, and liqueurs), which were adopted by many other countries (Conacher and Page, 1986; Abreu *et al*., 2005). In 1988 the US Food and Drug Administration (FDA) accepted a plan proposed by the largest American wineries and presented by the Wine Institute and the Association of American Vintners to reduce EC levels in table wines. The agreement stated that table wines (≤ 14º alcohol) from 1988 harvest should have a mean EC content below 15 μg/L (Butzke and Bisson, 1997; Uthurry *et al.*, 2004). In October 2006, European Food Safety Authority (EFSA) requests data and studies to the European Union (EU) member states about the presence of EC in foods and beverages. The EU includes EC within the group of contaminants, defined as those substances that have not been intentionally added to food and whose presence generally has a negative impact on the quality of food and may imply a risk to human health (European Commission Recomendation 2010/133/EU).

EC precursors are urea, produced by yeasts and citrulline and carbamyl phosphate produced by LAB that result from the catabolism of arginine.

In the last decades, several researches establish the biochemical mechanism used by yeasts and LAB to generate EC (Ingledew et al., 1987; Ough *et al.*, 1988, 1990; Monteiro *et al.*, 1989; Tegmo-Larsson *et al.*, 1989; Henschke and Ough, 1991; Kodama *et al.*, 1994; Liu *et al.*, 1994; Granchi *et al.*, 1998;

Arena *et al.*, 1999a, b; Mira de Orduña *et al.*, 2000, 2001). From a practical perspective, it was found that the physicochemical conditions used for wine ageing and storing have a particular effect on EC production (Kodama *et al.*, 1994).

For determination of EC, a number of practical gas chromatographic methods using a flame ionization detector (Kato *et al.*, 1989), hall electrolytic conductivity detector (Walker *et al.*, 1974; Dennis *et al.*, 1986), thermal energy analyzer with nitrogen converter (Canas *et al.*, 1989), mass spectrometer (Aylott *et al.*, 1987), high-resolution mass spectrometer (Lau *et al.*, 1987), or tandem mass spectrometer (Cairns *et al.*, 1987) have been used. Herbert *et al.* (2002) developed an HPLC method to determine EC in alcoholic beverages based on the reaction with 9-xanthydrol, which is a simple but reliable technique that may be easily used in laboratories with a large throughput of samples. In order to avoid the necessary extraction steps when GC is used, a selective reaction prior to HPLC separation was applied. In addition, because low detection levels are required, fluorescence detection was applied.

Based on several research results, FDA recommended require caution in the selection of starter cultures for conducting MLF in wine, since citrulline formation from arginine degradation could result in high levels of EC, even at normal temperatures, during prolonged storage. In addition, spontaneous MLF by undefined strains should be avoided, as this may lead to formation of EC precursors. If MLF is looked-for, winemakers should either use a selected strain that does not produce high levels of citrulline or monitor juice for citrulline content post-fermentation (Butzke and Bisson, 1997).

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CHAPTER 2

BIOGENIC AMINES IN WINE

This chapter focuses the study of the influence of oenological factors (winemaking region, grape variety, anti-fungi treatment of grapes, fermentation activators, malolactic starters and wine storage on lees) that could contribute for the production and presence of biogenic amines in wines.

This chapter consists of one scientific article:

Marques, A.P., Leitão, M.C., San Romão, M.V. 2008. Biogenic amines in wines: influence of oenological factors. Food Chemistry. 107: 853-860.

The experimental work presented in this chapter was done by the author. In the HPLC analysis the author had help of Maria Cristina Leitão. The manuscript was written by the author and revised by the other co-authors of the articles.

2.1 Biogenic amines in wines: influence of oenological factors

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Biogenic amines in wines: Influence of oenological factors

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Abstract

Biogenic amines formation results from the decarboxylation of the corresponding amino acids by action of microorganisms. The presence of these compounds is considered by some authors a fundamental parameter for detriment of alcoholic beverages. The aim of this work was to assay the effect of some oenological factors (viticulture region, grape variety, anti-fungi treatment of grapes, fermentation activators, malolactic starters and storage on lees) from the point of view of their influence on the biogenic amines content of wines. According to our results, it was possible to show that the viticulture region affects the amounts of amines, since wines of some regions present higher contents of amines than wines from other regions. Grape varieties appear to influence the wine amines content. Commercial malolactic starters, after careful selection, should be added to the vinification process in order to decrease the formation of biogenic amines, since in our assays the wines that were inoculated with starters present lower amounts of biogenic amines. The wine storage on lees contributes for a biogenic amines increase.

Keywords: Wine; Biogenic amines; Viticulture region; Grape variety; Anti-fungi treatment of grapes; Fermentation activators; Commercial malolactic starters; Storage on lees

1. Introduction

Biogenic amines (BA) are naturally occurring compounds, ubiquitous in animals and plants. They are low-molecular-weight organic bases, aliphatic (putrescine, cadaverine, spermidine and spermine), heterocyclic (histamine and tryptamine), or aromatic (tyramine and phenylethylamine) (Lounvaud-Funel, 2001). These active compounds play important roles in normal mammalian physiology, like cell proliferation and differentiation (Bauza et al., 1995). BA are formed by decarboxylation of the corresponding amino acids by microorganisms through substrate-specific decarboxylase enzymes. This property is not linked to a microbial species, usually it is strain dependent (Leitão, Teixeira, Barreto Crespo, & San Romão, 2000 and Moreno-Arribas, Polo, Jorganes, & Muñoz, 2003). This could, at least partially, explain why BA are randomly produced, in some wines they are detected sometimes presenting quite large values, while other ones show near trace values or do not present them at all. Decarboxylase enzymes are generally induced at acidic pH and therefore they have a possible role in maintaining pH homeostasis or enlarging the microbial growth period by detoxification of the extracellular medium (Marcobal, Rivas, Moreno-Arribas, & Muñoz, 2004 and Leitão et al., 2000).

The conditions that favour the occurrence of BA in wine dependent on time of must contact with grape skin, amino acid content at the initial and final phases of alcoholic fermentation and time of wine contact with yeast, (Vidal-Carou, Ambatlle-Espunyes, Ulla-Ulla, & Mariné-Font, 1990). The type and degree of ripeness of the grapes, the

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climate and soil of the viticulture area, and the vinification techniques also could contribute for the wine biogenic amines content (Ferreira & Pinho, 2006). BA in wine may have two different sources: raw materials and fermentation processes. Some amines are already found in grapes, namely histamine and tyramine (Vidal-Carou et al., 1990), as well as several volatile amines and polyamines (Feuillat, 1998).

Histamine, tyramine and putrescine are the BA found in higher concentration in wine, but cadaverine, phenylethylamine, isoamylamine can also be found (Bauza et al., 1995 and Silla Santos, 1996). Putrescine and cadaverine are normally associated with poor sanitary conditions of grapes (Leitão, Marques, & San Romão, 2005). Putrescine in grapevines has been also associated with potassium deficiencies in soil (Brodequis, Dumery, & Bouard, 1989). It is possible that this amine accumulates in the grapes, and consequently remains in the wine (Vidal-Carou et al., 1990 and Coton, Torlois, Bertrand, & Lonvaud-Funel, 1999).

The study of BA represents a concern for wine industry. From a toxicological point of view they can cause undesirable physiological effects in sensitive humans, especially if their metabolism is blocked or genetically altered (Ferreira & Pinho, 2006). They also can be a source of problems in commercial transactions since some countries have established maximum limits for histamine content in wine (Martín-Álvarez, Marcobal, Polo, & Moreno-Arribas, 2006).

The published studies concerning the toxicological effects in humans are contradictory. Some authors considered that the presence of BA in wine could be an important food safety problem due to some described implication of these compounds in cases of food intolerance and intoxication (Ferreira & Pinho, 2006; Martín-Álvarez et al., 2006; Marcobal, Martín-Álvarez, Polo, Muñoz, & Moreno-Arribas, 2006; Wantke, Gotz, & Jarisch, 1993, 1994, 1996). Under normal conditions, exogenous amines ingested as a part of the diet are absorbed and quickly transformed in the human organism by the action of the amine oxidases. However, when normal catabolic routes of amines are inhibited or a large amount of food containing BA is ingested, several physiological changes can occur, such as migraine headaches, nausea, hypo- or hypertension, cardiac palpitations, and anaphylactic shock (Silla Santos, 1996). According to other authors (Jansen, van Dusseldorp, Bottema, & Dubois, 2003; Kanny & Gerbaux, 2000; Kanny et al., 2001), no correlation was found between the occurrence of symptoms and the concentration of biogenic amines in wine samples, it appeared that red wine does not contain enough of key headache-producing compounds (histamine and tyramine) to be of significance in causing headaches and even psychosomatic reactions were admitted. Kanny et al. (1999) showed that the amount of histamine in wine has no clinical or biological effect in healthy subjects, and also emphasized the efficiency in man of the systems for degradation of histamine that is absorbed by the alimentary tract.

The wine industry is determined to reduce the presence of BA in wine. To better understand the prevention and control of the formation of these compounds, it is important to conduct critical analysis about the many factors associated with their development. The aim of this work was to study some oenological factors that could contribute to BA accumulation in wines.

2. Materials and methods

The oenological factors considered in this study were: viticulture region, grape cultivars, grape treated with different anti-fungi products, 2 fermentation activators (1 alcoholic fermentation activator and 1 malolactic fermentation activator), 2 commercial malolactic starters (CMS1 for red wines and CMS2 for white wines) and wine storage on lees. The wines used to study the influence of fermentation activators, commercial malolactic starters and storage on lees on BA concentration were produced on Estação Vitivinícola Nacional (EVN) during 2001 and 2002 harvest. The climatic conditions of 2001 and 2002 harvest were identical.

The wines were industrially elaborated in Portuguese wine-producing cellars. These wines were elaborated in stainless steel tanks following a typical red or white wine manufacturing process. The AF was carried out by indigenous yeast under controlled temperature.

At each sampling time, must and wine samples were collected and immediately frozen until analysis. Each assay was performed at least in duplicate and the mean values are reported.

2.1. Viticulture region

A total of 82 samples of red wines were produced in three different Portuguese regions (Douro, Dão and Alentejo) during 2003 and 2004 harvest. A total of 30 samples of red wines were elaborated in two wineries from Dão region on 2005 harvest.

2.2. Grape cultivars

The grape varieties used in this work were: Periquita, Espadeiro, Cabernet Sauvignon, Bastardo, Alfrocheiro and Tinta Miúda. The wines obtained from each cultivar were produced during the harvest of 1999.

2.3. Grape treated with different anti-fungi products

Only the wines from Periquita were used for this study during the 1999 harvest. In the vineyard, the cultivar Periquita was divided in 4 different groups, and each group was treated with a specific anti-fungi product. The fungicides tested were carbendazyme, iprodione and procymidone. The products were applied every 3 weeks in concentrations defined according to the climatic conditions observed. The 4th group had not received any anti-fungi treatment being considered the control group.

2.4. Fermentation activators

Two fermentation activators (nutritive factors) were tested: one activator of the alcoholic fermentation was added to the must and one activator of malolactic fermentation was added at the end of the AF. The fermentation activators (trade marks) were kindly supplied by the respective commercial supplier in Portugal.

2.5. Commercial malolactic starters

Two commercial malolactic starters were also tested (CMS1 in red wines; CMS2 in white wines) being added to the different wines at end of the AF, immediately after the first racking off. The malolactic starters (trade marks) were kindly supplied by the respective commercial supplier in Portugal. In these wines the MLF was conducted by the malolactic starters.

2.6. Storage on lees

At the end of MLF, the wines were divided in two parts. One part was stored on lees during 6 months in contact with the respective lees and the other one was stored for the same period without lees.

2.7. Biogenic amines analysis

Biogenic amines (histamine, tyramine, putrescine, cadaverine, phenylethylamine and isoamylamine) were analysed by reverse-phase high-pressure liquid chromatography (RP-HPLC) according to the method described by Vidal-Carou, Lazoh-Portolés, Bover-Cid, and Mariné-Font (2003). The RP-HPLC analysis was carried out with a fluorescence detector (excitation wavelength of 340 nm, and emission wavelength of 425 nm). The separations were performed on a Waters Nova-Pack C18 column. The derivatization process was post-column performed with *o*-phtaldialdehyde/2-mercaptoethanol (OPA/MCE) reagent. Samples were filtered (0.45 μm pore size filter; Millipore, USA) and then directly injected in duplicate onto the HPLC system. All the reagents used were HPLC grade.

3. Results

3.1. Viticulture region

Putrescine was the predominant amine in all of the analyzed wines (2004 harvest), at MFL end, from Douro, Dão and Alentejo viticulture regions. The wines from Douro and Alentejo presented the higher mean amounts of putrescine (10.9 ± 6.8 mg/L and 17.3 ± 5.0 mg/L, respectively), tyramine (2.8 ± 2.2 mg/L and 2.0 ± 1.8 mg/L, respectively) and histamine (5.0 ± 2.9 mg/L in both regions). The amounts of cadaverine, isoamylamine and phenylethylamine were always low. The wines from Dão presented the lowest levels of BA and phenylethylamine was not detected in those wines (data not shown).

Wines from two wineries both in the same region (Dão) were studied, in order to determine the differences in BA. Wineries from the same region present different amounts of these organic compounds, especially, tyramine and putrescine. The amounts of cadaverine, isoamylamine and phenylethylamine never exceeded 1 mg/L. The wines produced in winery A present higher mean concentration values of tyramine (12.0 ± 2.3 mg/L) than wines from winery B (0.5 ± 0.1 mg/L of tyramine). The wines from winery B showed slightly higher amounts of putrescine than wines from winery A (5.0 ± 1.9 mg/L and 2.0 ± 0.9 mg/L, respectively). The amounts of histamine were identical in both wineries. Phenylethylamine was not detected in wines from winery B (data not shown).

3.2. Grape varieties

At the end of AF only small amounts of BA were detected in the several wines (data not shown). After MLF, in wines obtained from the 6 different grape varieties tyramine was the BA present in higher concentration. At the end of MLF, tyramine clearly increased especially in wines arising from the cultivars Alfrocheiro (31.5 ± 6.2 mg/L) and Espadeiro (24.4 ± 4.5 mg/L) (Fig. 1). The three wines obtained from Espadeiro, Bastardo and Alfrocheiro revealed low quantities of isoamylamine (between 2.5 ± 0.9 mg/L and 6.2 ± 2.1 mg/L). The wines arising from Piriquita, Cabernet Sauvignon, Bastardo and Tinta Miúda presented low levels of BA. The levels of cadaverine, histamine, phenylethylamine and putrescine were always low in all the wines.

3.3. Grape treated with different anti-fungi products

At the end of alcoholic fermentation no significant amounts of BA were detected in all the wines (data not shown). In this phase of the vinification process the levels of these compounds never exceeded 0.5 mg/L. After MLF achievement the control wines presented, on the whole, higher mean concentrations value of BA, than wines obtained from grapes treated with fungicides especially in the cases of isoamylamine (11.6 ± 0.6 mg/L), phenylethylamine (3.5 ± 0.4 mg/L) and tyramine (6.4 ± 5.0 mg/L), (Fig. 2). The wines from grapes treated with different fungicides present contents of tyramine between 1.7 ± 0.4 mg/L and 2.5 ± 0.3 mg/L, isoamylamine between 1.0 ± 1.2 mg/L and 4.1 ± 2.1 mg/L and the other BA never exceed 1.6 mg/L. The wines from grapes treated with carbendazyme presented the higher contents of BA and the wines from grapes treated with procymidone showed the lower levels of these compounds.

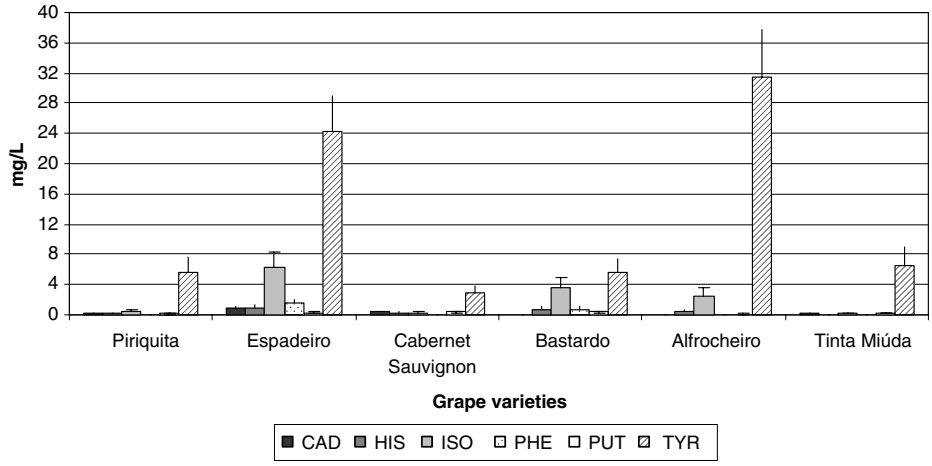

Fig. 1. Biogenic amines (mg/L) in wines produced from different grapes cultivars, at MLF end. Results are expressed as mean values ± standard errors. ■ Cadaverine; ■ histamine; ■ isoamylamine; ⊡ phenylethylamine; and □ putrescine ▨ tyramine.

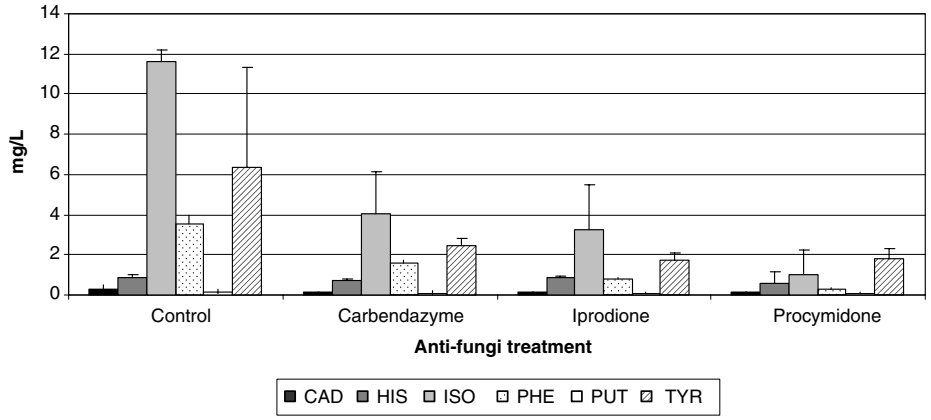

Fig. 2. Biogenic amines (mg/L) in wines from grapes treated with anti-fungi products, at MLF end. Results are expressed as mean values ± standard errors. ■ Cadaverine; ■ histamine; ■ isoamylamine; ⊡ phenylethylamine; □ putrescine; and ▨ tyramine.

3.4. Fermentation activators

The BA concentrations on wines added or not with the alcoholic fermentation activator are shown in Fig. 3a and those added or not with the malolactic fermentation activator are shown in Fig. 3b. Isoamylamine and tyramine were the BA present in higher concentration in must. Cadaverine, histamine, phenylethylamine and putrescine are present in concentration lower than 1 mg/L. At the end of AF a slight increase in tyramine and phenylethylamine, and a decrease in isoamylamine were observed. At the end of MLF, a slight formation of tyramine can be noticed. The amounts of cadaverine, histamine, phenylethylamine and putrescine were always very low.

The wines resulting from the addition of AF activator presented amounts of isoamylamine and tyramine slightly higher than wines not added, especially at the end of MLF (Fig. 3a). The wines added with the MLF activator, presented similar amounts of BA than the control wines (Fig. 3b).

3.5. Commercial malolactic starters

The malolactic starter CMS1 was only tested in red wines according to the manufacturer instructions. Two months after MLF was finished, the inoculated wines presented lower concentration of BA than the control wines (Fig. 4a). Tyramine (18.9 ± 2.2 mg/L), putrescine (4.4 ± 2.4 mg/L) and histamine (4.3 ± 0.07 mg/L) were the BA present in higher concentration in control wines. For the inoculated wines only tyramine exceeds the 10 mg/L, while all the other ones presented very low levels.

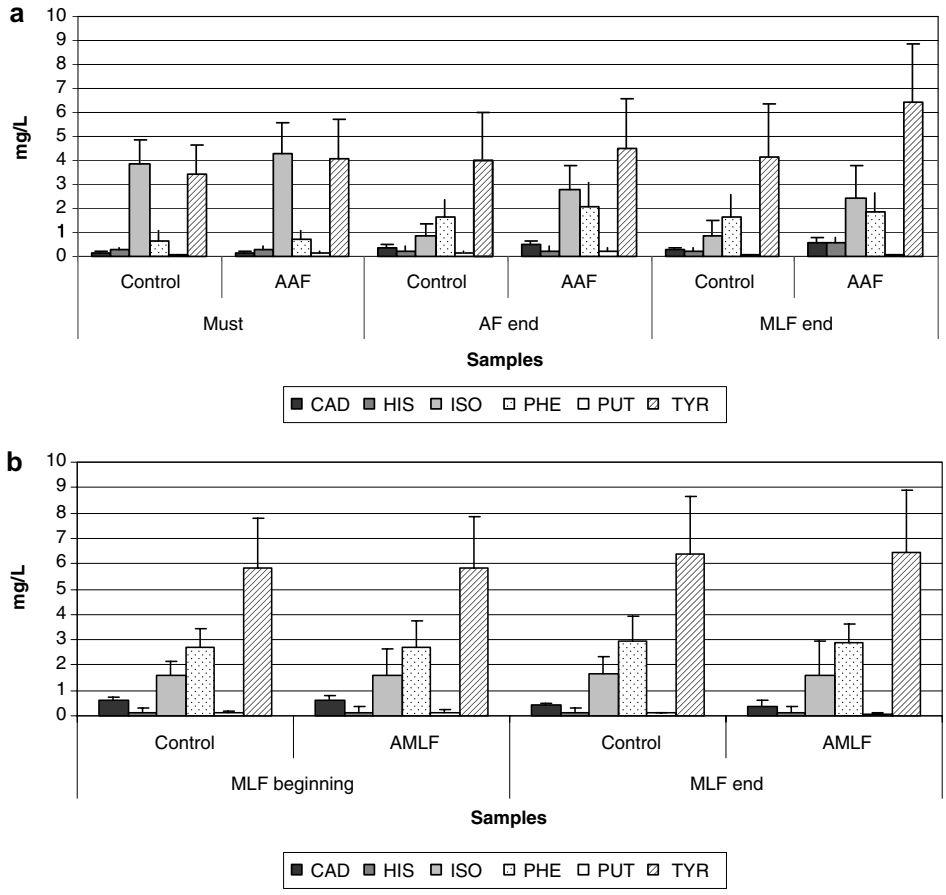

Fig. 3. Biogenic amines (mg/L) in wines added and not added with fermentation activators. (a) Wines added and not added with alcoholic fermentation activator. Sampling on Must, end AF and end MLF; and (b) wines added and not added with malolactic fermentation activator. Sampling on MLF beginning and MLF end. (AAF: wines added with AF activator; AMLF: wines added with MLF activator). Results are expressed as mean values ± standard errors. ■ Cadaverine; ■ histamine; ■ isoamylamine; □ phenylethylamine; □ putrescine; ▨ tyramine.

The malolactic starter CMS2 was tested in white wines also according to the producer instructions. At the end of MLF the control wines presented higher mean concentrations value of tyramine (7.0 ± 2.2 mg/L) than the inoculated wines which presented 2.0 ± 2.0 mg/L (Fig. 4b). In this assay, two months after MLF was accomplished, the amount of tyramine and cadaverine in control wines increased while in inoculated wines only tyramine increased although the detected levels were always lower than in the control wines.

3.6. Storage on lees

As stated above, a portion of the red wine was stored for six months without lees and another portion was stored for the same period in presence of the respective lees. Two months after MLF the BA levels of histamine, isoamylamine, phenylethylamine and putrescine were identical in both assays (Fig. 5). The tyramine and cadaverine levels were higher for wines stored on lees. Six months after MLF was possible to observe a slight increase of tyramine (from 6.3 ± 2.0 mg/L to 7.8 ± 2.4 mg/L) and a decrease of cadaverine in wines stored on lees. In the case of the control wines it was possible to observe that tyramine content increased markedly from ca. 0.2 to 5.5 mg/L after 6 months of storage, probably due to the presence of high producing-tyramine bacteria.

4. Discussion

Wine can be an ideal substrate for BA production, because its manufacturing process involves available free amino acids, the possible presence of decarboxylase-positive microorganisms, and some favorable environmental conditions that affect the growth of microorganisms and the activity of decarboxylase enzymes (Lounvaud-Funel,

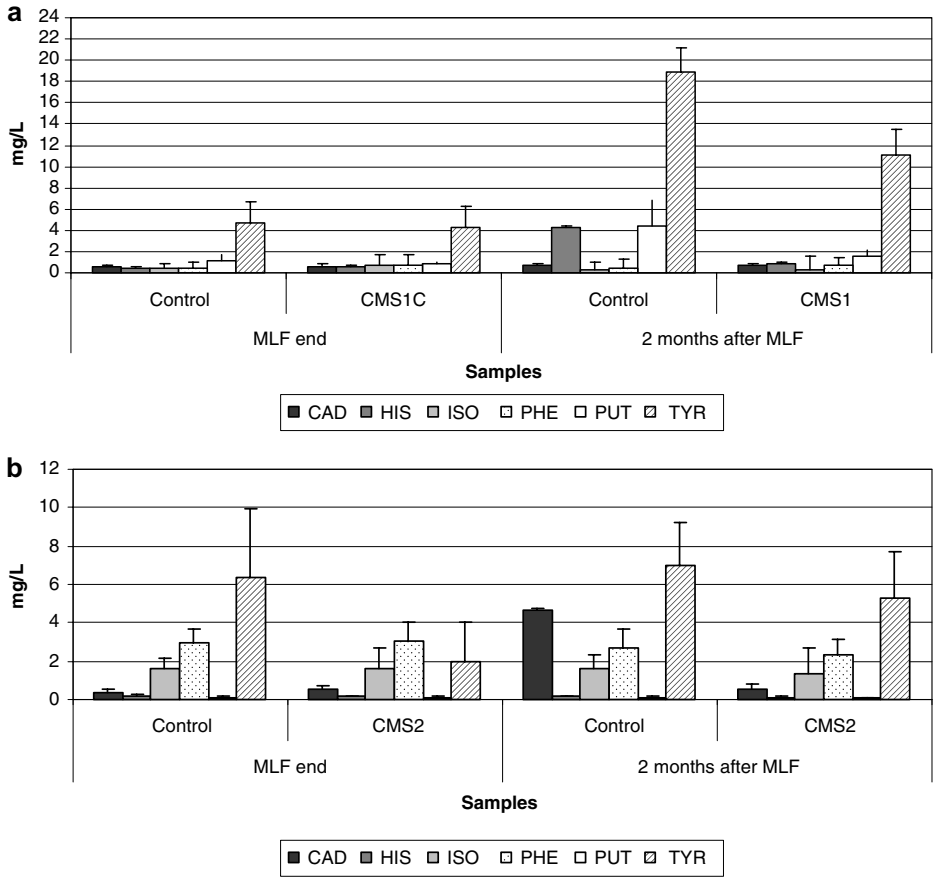

Fig. 4. Biogenic amines (mg/L) in wines added and not added with commercial malolactic starters. (a) Wines inoculated and not inoculated with commercial malolactic starter 1 (CMS1). Sampling: MLF end and 2 months after MLF; and (b) wines inoculated and not inoculated with commercial malolactic starter 2 (CMS2). Sampling: MLF end and 2 months after MLF. Results are expressed as mean values ± standard errors. ■ Cadaverine ■ histamine; ■ isoamylamine; ⊡ phenylethylamine; □ putrescine; and ▨ tyramine.

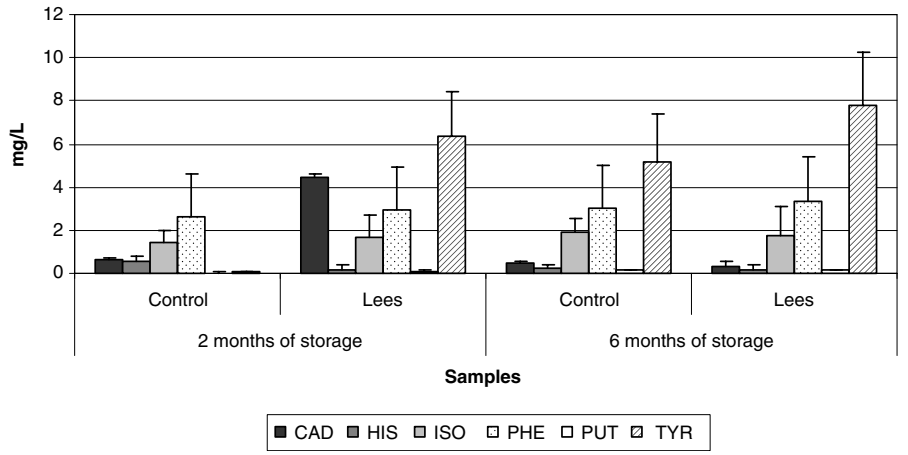

Fig. 5. Biogenic amines (mg/L) in wines storage and not storage on lees. Sampling: 2 months after MLF and 6 months after MLF. Results are expressed as mean values ± standard errors. ■ Cadaverine; ■ histamine; ■ isoamylamine; ⊡ phenylethylamine; □ putrescine; and ▨ tyramine.

2001). The definition of wine quality should have in attention its BA contents mainly due to commercial interests. The regulatory limits for BA in wines have not yet been established by OIV (“Organization International de la Vigne et du Vin”), however some countries had create maximum limits for histamine content in wine (2 mg/L in Germany, 6 mg/L in Belgium, 10 mg/L in Switzerland and Austria, 8 mg/L in France and 4 mg/L in Holland) (Busto, Guasch, & Borrull, 1996). The maximum limit of biogenic amines generally considered to be safe for consumers is 10 mg/L (Loret, Deloyer, & Dandrifosse, 2005).

The major goal of this work was to study the effect of some oenological factors (viticulture region, grape variety, anti-fungi treatment of grapes, alcoholic fermentation activator, malolactic fermentation activator, commercial malolactic starter and storage on lees) on biogenic amines levels.

In the last few years it was possible to observe an increased of BA levels in Portuguese wines (Leitão et al., 2005). This can be attributed to climatic conditions, sanitary conditions of grapes, vinification method and or dominance of LAB species in the must.

Wines produced in two wineries from Dão region present different amounts of BA what is not surprising once the grapes origin, sanitary state and also winemaking process as well as indigenous microorganisms should be different.

Tyramine and isoamylamine were the BA present in higher amounts in wines obtained from 2003 harvest while in 2004 putrescine was the most important BA. The observed differences in BA in wines from different years can be due to the diversity of wine microorganisms that are naturally differently selected each year, probably due to climatic conditions and consequent viticulture/oenological practices. The observed difference in BA content between grape varieties is probably due to inherent types of amino acids composition and respective amounts in grape varieties. Other hypothesis to explain this difference is the natural bacteria microflora present on grapes.

The obtained results from anti-fungi treatment assays clearly show that fungal metabolic activity could have some influence in biogenic amine formation (especially isoamylamine) since, control wines made with grapes not treated with anti-fungi products present higher content of BA than wines obtained from treated grapes. To our knowledge, only a few studies reported information about the presence of amines in musts (Bertoldi, Larcher, & Nicolini, 2004 and Marcobal et al., 2006). The obtained results suggest that BA origin can be attributed either to the development of fungi in non treated grapes or to the activity of bacteria other than those normally present in healthy grapes.

In this study it was possible to detect isoamylamine and tyramine in the analyzed musts. Isoamylamine is normally associated to the activity of Enterobacteriaceae and fungi (Tavakkol & Drucker, 1983). At the end of AF the amounts of isoamylamine and tyramine showed a slight decrease when compared to the must contents, probably due to a co-precipitation with fine lees.

The addition of the AF and MLF activators to the wines does not appears to have any influence on BA formation, since those wines present almost the same amounts of BA as the control ones.

This study also confirmed that red wines present higher levels of BA than white wines, which can be explained by the different wine making techniques. On the contrary to white wines, red wines are prepared in presence of grape skins and grapes are submitted to some pressing. These practices tend to induce higher nutrients and microorganisms extraction in red musts. Therefore microbial activity is favoured and the obtained results are not surprising.

Previous works showed that some commercial malolactic bacteria did not produce BA (Moreno-Arribas et al., 2003). This work demonstrated that the application of commercial malolactic starters in wines was useful to reduce the BA amounts, since in the inoculated wines BA concentrations were significantly lower when compared with those not inoculated. In the case of the not inoculated wines the amounts of BA were higher, probably because MLF was conducted by indigenous malolactic bacteria higher BA producers. These results suggest that the use of well selected malolactic starters can minimise biogenic amines production.

Wine storage in presence of fine lees appears to contribute for the increase of BA in those wines. This was probably due to the contact of the wine with lees during which the proteins present in lees, mainly resulting from yeast autolysis, are hydrolysed to peptides and these peptides are later degraded further to amino acids and amines. These results are in agreement with those reported by Bauza et al. (1995).

From the overall results obtained along this study it is possible to conclude that the majority of the wines obtained appear to be safe either from a healthy or from a “legal” point of view as the levels of BA were normally low (<10 mg/L).

Additional investigation should be performed to understand and establish limits for biogenic amines levels in commercial wines. Control measures are needed to prevent BA formation keeping their levels in wine as low as possible what should obviously start by the choice of the lactic acid bacteria to be used as MLF starters. Also, further toxicological studies should be performed in order to analyze the potential impact of these compounds in human health.

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CHAPTER 3

PORTUGUESE AUTOCHTHONOUS *OENOCOCCUS OENI* STRAINS

The first part of this Chapter is dedicated to the development of a new molecular method for the identification of *Oenococcus oeni* and its specific detection in wine, based on the amplification of 16S rRNA gene followed by restriction with the endonuclease FseI. In the second part of Chapter III, 121 *O. oeni* strains were isolated from wines of different winemaking regions of Portugal and identified by a phenotypic and molecular approach. M13-PCR fingerprinting analysis was thus carried out to evaluate the genetic diversity of this collection of *O. oeni* strains and search for underlying patterns of regional/geographical strain diversity. The third part of Chapter III covers the selection of Portuguese autochthonous *O. oeni* strains to be used as malolactic starters on the wine industry.

This chapter consists of two scientific articles:

Marques, **A.P.**, Zé-Zé, L., San Romão, M.V., Tenreiro. R. 2010. A novel molecular method for identification of *Oenococcus oeni* and its specific detection in wine. International Journal of Food Microbiology. 142(1-2): 251:255.

Marques, **A.P.**, Duarte, A.J., Chambel, L., Teixeira, M.F., Tenreiro, R., San Romão, M.V. 2011. Genomic diversity of *Oenococcus oeni* from different viticulture regions of Portugal. International Microbiology. 14: 155-162.

The experimental work presented in this chapter was done by the author. In the M13-PCR fingerprinting analysis and growth studies of *O. oeni* the author had help of Ana Judite Duarte and Liliana Pinto, respectively. The manuscript was written by the author and revised by the other co-authors of the articles.

3.1 A novel molecular method for identification of *Oenococcus oeni* and its specific detection in wine

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Short communication

A novel molecular method for identification of *Oenococcus oeni* and its specific detection in wine

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ABSTRACT

Oenococcus oeni is a species of lactic acid bacteria with economic interest in winemaking. Using both *in silico* and *in vitro* analyses, a molecular method was developed that allows the identification of *O. oeni* isolates and its detection from wine samples. The method is based on the amplification of 16S rRNA gene with universal primers followed by restriction with the endonuclease *Fse*I, generating two fragments of 326 and 1233 bp. Among wine bacteria, the *Fse*I recognition sequence is only found in the 16S rRNA gene of *O. oeni*, ensuring the specificity of the method. The use of Whatman FTA cards for DNA extraction and purification is an efficient and interesting alternative to current methods, as samples can be easily collected at wineries by a non-specialized technician, stored at room temperature and sent in a mail envelope to the analytical laboratory for processing. The proposed method, with a detection limit between 10^2 and 10^3 cfu/mL and a full turnaround time of *ca.* 8 h, ensures the rapid and reliable detection of *O. oeni* in wine samples during winemaking surveillance and wine quality control.

1. Introduction

Lactic acid bacteria (LAB), belonging to genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*, are present in grape musts with populations that vary from 10^2 to 10^4 cfu/mL. Along alcoholic fermentation in winemaking, a natural selection occurs driven by the low pH, the grape sulfiting level and the increase in alcohol concentration and *Oenococcus oeni*, a heterofermentative coccus, usually becomes the dominant LAB species mainly due to its high tolerance to acidic conditions and ethanol (Costello et al., 1983; Lafon-Lafourcade et al., 1983). After alcoholic fermentation *O. oeni* populations start to multiply to levels of 10^6–10^8 cfu/mL and carry out the malolactic fermentation (MLF), a decarboxylation process driven by the malolactic enzyme (EC 1.1.1.38) that converts L-malic acid into L-lactic acid (Lonvaud et al., 1977). MLF is an important step in the vinification process as it ensures deacidification and microbial stability of red and white wines and its extent will depend on medium conditions (Bousbouras and Kunkee, 1971; Davis et al., 1988; Renouf et al., 2006a; Ribereau-Gayon et al., 2006a). Besides wine deacidification, MLF also enhances organoleptic properties, contributing to wine sensory quality (de Revel et al., 1999; Lonvaud-Funel, 1999).

After malic acid consumption, by *O. oeni*, the remaining LAB present in wine need to be inactivated, even removed, as due to the pH increase after malic acid catabolism they are able to develop and metabolise other substrates being at the origin of wine depreciation (Ribereau-Gayon et al., 2006b). As a consequence, an accurate control of malic acid and/or total LAB population is required along the winemaking process.

LAB and *O. oeni* in particular are difficult to study and characterise, as they are quite fastidious implying much time-consuming work often leading to ambiguous results. Several molecular approaches have been applied to detect and/or identify wine LAB, including dot-blot or colony hybridization with specific DNA probes (Lonvaud-Funel et al., 1991), PCR amplification of *mle* gene with specific primers (Zapparoli et al., 1998), amplification and sequencing of 16S rRNA or *rpo*B genes (Renouf et al., 2006a,b; Sato et al., 2001), 16S rRNA- or *rpo*B-based analysis of restriction fragment length polymorphisms (Claisse et al., 2007; Sato et al., 2000), analysis of wine microbial communities by PCR-DGGE (Lopez et al., 2003; Renouf et al., 2006a,b; Spano et al., 2007), fluorescent in-situ hybridization (Blasco et al., 2003) and real-time quantitative PCR (Pinzani et al., 2004). Overall, molecular biology techniques are useful for the quick and accurate identification of these microorganisms, opening the road for their use in winemaking surveillance and wine quality control.

The present work concerns the development of a new molecular method for the identification of *O. oeni* and its specific detection in wine, based on the amplification of 16S rRNA gene followed by restriction with the endonuclease *Fse*I. Among wine bacteria, the *Fse*I recognition sequence is only found in the 16S rRNA gene of *O. oeni*, thus ensuring the

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specificity of the method. Furthermore, the DNA extraction and purification procedure rely on the use of Whatman FTA cards, a patented technology for handling and processing of nucleic acids with a validated application in a wide range of fields (forensics, biomedicine, pharmaceutics, genomics, and food testing). The proposed application for wine samples is a novelty and an efficient and interesting alternative to current DNA wine DNA extraction methods, since samples can be easily collected at wineries by a non-specialized technician, stored at room temperature and sent in a mail envelope to the analytical laboratory for direct PCR and amplicon restriction analysis.

2. Materials and methods

2.1. Microorganisms, growth conditions and wine samples

Fifteen *O. oeni* strains, isolated from wines of West Ribatejo region of Portugal at the end of MLF, and belonging to the IBET culture collection were used in this study. The identification of these isolates at species level was confirmed by phenotypic and genomic fingerprinting methods. A commercial malolactic starter from Christian Hansen (Viniflora oenos) and the *O. oeni* type strain (DSMZ20252^T) were also included. All strains were cultured in MTJ broth medium (50% MRS broth, Merck, Germany; 50% Tomato Juice broth, Difco, USA) (pH 5.5) at 30 °C until the stationary phase.

To assay the detection limit of the molecular technique, Viniflora oenos was inoculated in a sterile wine at a concentration of 10^6 cfu/mL, 10-fold serial dilutions were made using sterile wine and the number of viable cells was determined for each dilution by standard colony counting on MTJ agar plates (pH 5.5; 1.2% agar).

Samples from ten red wines undergoing MLF and three finished white wines, kindly provided by ex-Estação Vitivinícola Nacional (EVN, Dois Portos, Portugal), were used to evaluate the application of this method. For wine samples, data on microbial counts were also available from EVN.

2.2. DNA extraction

DNA extraction from broth cultures, inoculated sterile wines and red and white wine samples was performed using the DNA storing and extraction FTA cards (Whatman, USA). For each sample, an aliquot of 150 µl was applied on the FTA card that was allowed to dry completely at room temperature. A 2 mm disk was punched out from the FTA card, using the Harris Uni-Core punch and the cutting mat provided by the manufacturer, and placed in a 0.2 mL PCR tube. The disk was washed twice with the FTA purification reagent, discarding it after each wash. After washing the disk twice with TE buffer (10 mM Tris, 0.1 mM EDTA, and pH 8.0), discarding the used buffer after each wash, the disk was left to dry in the PCR tube until its use for PCR amplification.

2.3. 16S rRNA gene amplification and restriction analysis (ARDRA)

The 16S rRNA gene was amplified by PCR using the universal primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′) (Ulrike et al., 1989). To the PCR tube containing 1 disk of FTA card, treated as described above, 50 µl of a reaction mixture containing 1× Taq polymerase buffer (Invitrogen, USA), 200 µM deoxynucleoside triphosphate mix (Invitrogen, USA), 3 mM magnesium chloride (Invitrogen, USA), 1 µM of each primer (MWG, Germany), 1× BSA (New England Biolabs, USA), and 0.1 U of Taq DNA polymerase (Invitrogen, USA) were added. Amplification was carried in a thermal cycler (Personal Cycler; Biometra) under the following conditions: 10 min of initial denaturation at 95 °C, then 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The amplification was completed by holding for 5 min at 72 °C to allow complete extension of the PCR products. The amplification products were visualized by ethidium bromide staining after electrophoresis through 1% (w/v) agarose (Seakem, BMA, USA) gel.

The restriction analysis was performed using *Fse*I (New England Biolabs, USA). Each 16S rDNA amplicon (5 µl) was digested with 0.5 U of *Fse*I, with 1 µl of the corresponding enzyme buffer (10× concentrated) in a final volume of 10 µl by 3 h incubation at 37 °C. The DNA restriction fragments were analysed by electrophoresis in a 1% (w/v) agarose (Seakem, BMA, USA) gel and visualized by ethidium bromide staining.

2.4. Sequence data analysis

To assess the use of *Fse*I ARDRA assay for *O. oeni* identification, 16S rDNA sequences of indigenous wine bacteria and other phylogenetic related species were retrieved from public database Entrez at NCBI webpage (http://www.ncbi.nlm.nih.gov/entrez) and sequences were scanned for the presence of *Fse*I restriction site. Sequences were previously imported to BioEdit Sequence Alignment Editor version 4.8.6 (Hall, 1999) and alignments were performed using CLUSTAL W (Thompson et al., 1994).

3. Results and discussion

The cleavage of *O. oeni* 16S rRNA gene by the endonuclease *Fse*I was already known since the construction of *O. oeni* PSU-1 physical map (Zé-Zé et al., 1998) and further confirmed with the construction of physical maps for eight *O. oeni* strains selected as representatives of two genomic divergent groups (Zé-Zé et al., 2000, 2008).

The specificity of *Fse*I restriction for *O. oeni* 16S rRNA gene was investigated by *in silico* comparative sequence analysis of 16S rRNA genes from 22 species of the genera *Leuconostoc*, *Weissella*, *Lactobacillus* and *Pediococcus*, including the lactic acid bacteria currently found in wine as well as their phylogenetic closest neighbours. The *Fse*I recognizing sequence (GGCCGGCC) was exclusive of *O. oeni* and located at *ca.* 300 nt of the 5′ end of 16S rRNA gene, with one to three A or T nucleotides being found in the first bases of the recognition site in other LAB species (Fig. 1A).

To further assess the specificity of the *Fse*I recognizing sequence, the *in silico* analysis was extended and a survey of this sequence was performed on the Ribosomal Database Project (http://rdp.cme.msu.edu). Interestingly, the *Fse*I recognition site was found conserved in this position only in *O. oeni* and *O. kitaharae*, a recently new described species isolated in Japan from a composting distilled shochu residue (Endo and Okada, 2006), revealing its molecular marker potential for the genus *Oenococcus*. Although the *Fse*I recognition site was also found in the 16S rRNA gene of some genera of gram-negative and gram-positive bacteria, its position at *ca.* 200 or 1400 nt from the 5′ end of this gene was clearly different from *Oenococcus*, confirming the specificity of the *Fse*I restriction profile. Furthermore, no other described wine bacteria, including the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* of acetic acid bacteria, were found to harbour this recognizing sequence in their 16S rRNA gene.

Therefore, an amplified ribosomal DNA restriction analysis (ARDRA) was developed to specifically detect and identify *O. oeni*, based on the amplification of 16S rRNA gene with universal primers followed by direct amplicon restriction with *Fse*I.

The application of the ARDRA technique for identification of *O. oeni* isolates was tested with the type strain DSMZ 20252^T, one commercial malolactic starter (Viniflora oenos, Christian Hansen) and 15 *O. oeni* isolates obtained from Portuguese wines. For all *O. oeni* strains and isolates, a 16S rDNA amplicon with an observed size of *ca.* 1560 bp was obtained using primers pA and pH and two fragments with 326 and 1233 bp were observed after restriction with *Fse*I (as illustrated in Fig. 1B), as expected from the *in silico* analysis. Although the 3-hour incubation with 0.5 U of *Fse*I is insufficient for a complete digestion, since the unrestricted amplicon of *ca.* 1560 bp is still visualized in Fig. 1B, this procedure eliminates the need for a previous electrophoresis for

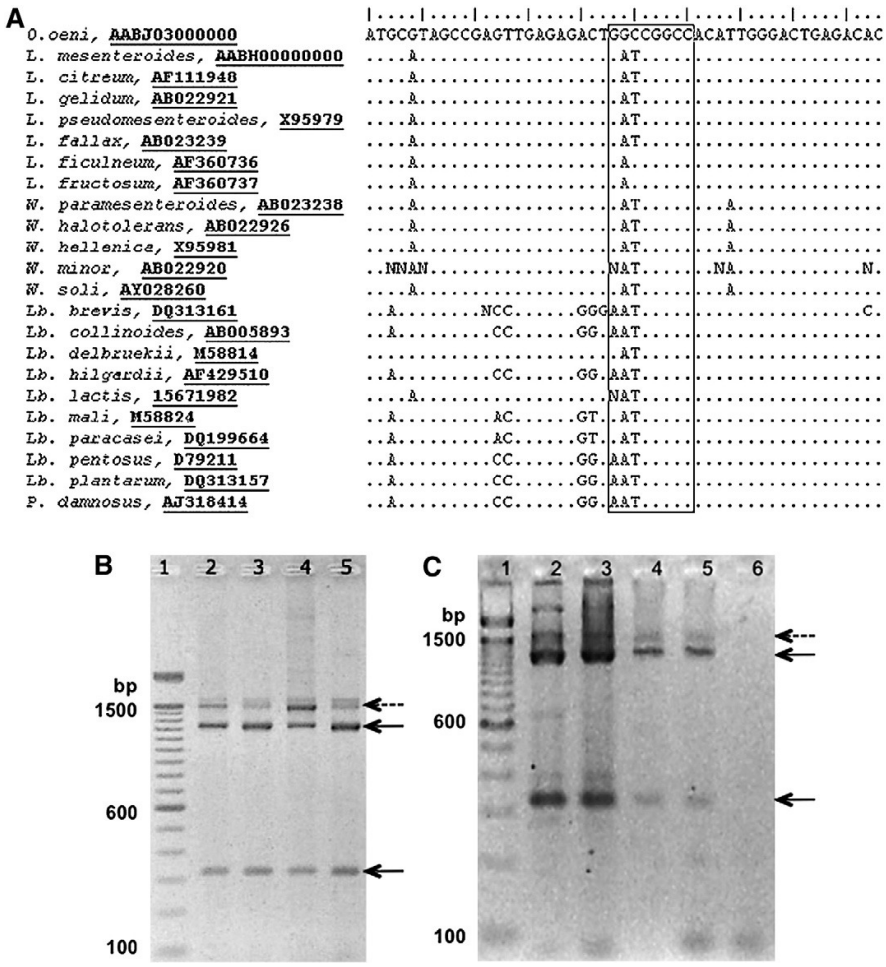

Fig. 1. (A) Partial sequence alignment of several LAB 16S rRNA gene sequences in the *Fse*I recognition site region of *Oenococcus oeni* sequence. The *Fse*I recognition site divergence is highlighted in a box. Species and GenBank accession numbers are shown at the right side of each sequence. *O.*, *Oenococcus*; *L.*, *Leuconostoc*; *W.*, *Weissella*, *Lb.*, *Lactobacillus*; *P.*, *Pediococcus*. (B) ARDRA analysis of the 16S rRNA gene from *Oenococcus oeni* strains and isolates using FTA cards and *Fse*I. Lane 1: 100 bp molecular marker (Invitrogen); lane 2: Viniflora oenos; lane 3: DSMZ 20252^T; lane 4: IO1; lane 5: IO24. (C) ARDRA analysis of the 16S rRNA gene amplified from wine samples, spiked with the commercial malolactic starter Viniflora oenos, using FTA cards and *Fse*I. Lane 1: 100 bp molecular marker; lane 2: wine spiked with 10^6 cfu/mL; lane 3: wine spiked with 10^5 cfu/mL; lane 4: wine spiked with 10^4 cfu/mL; lane 5: wine spiked with 10^3 cfu/mL; lane 6: wine spiked with 10^2 cfu/mL. Solid arrows indicate the 326 and 1233 bp *O. oeni* specific restriction fragments. Dashed arrow indicates the 1560 bp unrestricted amplicon.

confirmation of the PCR product, saves time to obtain the final result and reduces costs with this expensive enzyme, without any loss of information or reliability.

Direct detection of *O. oeni* in wine was also investigated using spiked wine samples and red and white wines. To check the sensibility of the ARDRA technique, the commercial malolactic starter Viniflora oenos was inoculated in a sterile wine sample (10^6 cfu/mL), serial decimal dilutions (up to 100 cfu/mL) were made and one disk from FTA cards was submitted to 16S rRNA gene amplification and subsequent *Fse*I restriction. As depicted in Fig. 1C, the molecular markers for the presence of *O. oeni* (fragments with 326 and 1233 bp) were detected in the spiked wine samples with up to 10^3 cfu/mL, no amplification product being obtained for the last decimal dilution (100 cfu/mL). Hence, the detection limit of this method in wine is estimated to be higher than 10^2 cfu/mL and lower than 10^3 cfu/mL.

The efficiency of the technique was checked using ten red and three white EVN wines. Although a 16S rRNA gene amplification product with *ca.* 1560 bp was detected with the universal primers pA and pH in all EVN wines, confirming the presence of bacteria (*O. oeni* and/or other species) and excluding the possibility of false negatives in the PCR step, the molecular markers for *O. oeni* were only detected in eight red wines (Fig. 2).

These results correlated well with the microbiological analysis of the wine samples. In fact, no oenococcus were detected by colony plate count ($<10^2$ cfu/mL) both in the white wines and the two negative red wines, although other bacteria and yeasts were detected, the former justifying the 16S rRNA gene amplicons. Regarding the positive red wines, the level of oenococcus varied from 2×10^2 to $>10^4$ cfu/mL. These findings give support to the detection limit estimated with the spiked samples and are also in accordance with the most current oenological practices since, except for some variety and wine regions (*v.g.* Chardonnay in Burgundy, Chasselas in Switzerland, German and Austrian varieties) where deacidification and/or aroma modulation is desired, malolactic fermentation is usually not practiced for white wines (Lonvaud-Funel, 1999; Ribereau-Gayon et al., 2006a).

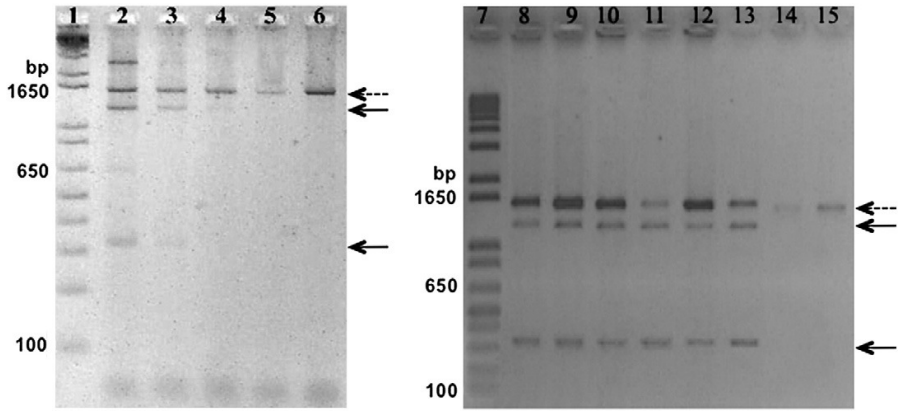

Fig. 2. ARDRA analysis of the 16S rRNA gene amplified from EVN wines using FTA cards and *Fse*I. Lanes 1, 7: 1 kb plus molecular marker (Invitrogen); lanes 2, 3, 8–15: red wines; lanes 5, 6: white wines. Solid arrows indicate the 326 and 1233 bp *O. oeni* specific restriction fragments. Dashed arrow indicates the 1560 bp unrestricted amplicon.

The use of Whatman FTA cards provides an easy way to purify genomic DNA from biological samples for PCR-based genetic analysis. In fact, FTA cards contain chemicals that lyse cells (both prokaryotic and eukaryotic), denature proteins and protect nucleic acids from nucleases, oxidative, and UV damage. The nucleic acids are released, entrapped in the fibers of the card matrix and remain immobilized, being automatically stabilized without the need of refrigeration. DNA is purified on the FTA card in three simple steps and in a single tube, the whole procedure taking no more than 30 min at room temperature. Removal of potential inhibitors by plug washing steps, combined with the use of BSA in the amplification mixture, ensures positive amplifications producing enough DNA for restriction analysis both from cell suspensions, spiked EVN wines (Fig. 1C) and non spiked wine samples (Fig. 2). Although several methods were described for DNA extraction from wines, either developed in-house or based on commercial kits (Jara et al., 2008; Pinzani et al., 2004; Renouf et al., 2006b), the FTA cards are an efficient and more interesting alternative since the samples can be easily collected at wineries by a non-specialized technician, stored at room temperature and sent in a mail envelope to the analytical laboratory for processing.

The ARDRA method here described, with a full turnaround time of *ca.* 8 h, is a rapid, sensitive and reliable technique for the positive identification of *O. oeni* isolates and could be considered a valuable tool for monitoring *O. oeni* in wine, as it can and reliably make the identification/detection of *O. oeni* in winemaking surveillance. Nevertheless, a more exhaustive evaluation of its applicability should be performed with a wider and more diverse range of wines, as well as by testing samples along the MLF process, to evaluate its usefulness for routine analysis in wine quality control laboratories. Moreover, as FTA cards allowed the extraction of nucleic acids free of PCR inhibitors from wine samples, their use in other PCR-based protocols (*e.g.* PCR-DGGE for wine microbiota analysis, PCR detection of yeasts or bacteria with specific primers, RT-PCR) seems also promising.

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3.2 Genomic diversity of *Oenococcus oeni* from different winemaking regions of Portugal

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Genomic diversity of *Oenococcus oeni* from different winemaking regions of Portugal

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Summary. *Oenococcus oeni* is an alcohol-tolerant, acidophilic lactic acid bacterium that plays an important role in the elaboration of wine. It is often added as a starter culture to carry out malolactic conversion. Given the economic importance of this reaction, the taxonomic structure of this species has been studied in detail. In the present work, phenotypic and molecular approaches were used to identify 121 lactic acid bacteria strains isolated from the wines of three winemaking regions of Portugal. The strains were differentiated at the genomic level by M13-PCR fingerprinting. Twenty-seven genomic clusters represented by two or more isolates and 21 single-member clusters, based on an 85% similarity level, were recognized by hierarchic numerical analysis. M13-PCR fingerprinting patterns revealed a high level of intraspecific genomic diversity in *O. oeni*. Moreover, this diversity could be partitioned according to the geographical origin of the isolates. Thus, M13-PCR fingerprint analysis may be an appropriate methodology to study the *O. oeni* ecology of wine during malolactic fermentation as well as to trace new malolactic starter cultures and evaluate their dominance over the native microbiota. [**Int Microbiol** 2011; 14(3):155-162]

Keywords: *Oenococcus oeni* · lactic acid bacteria (LAB) · Portuguese winemaking regions · genomic diversity · M13-PCR · fingerprinting

Introduction

In the mid 1960s, Ellen Garvie [13] isolated, characterized, and named *Leuconostoc oenos* as the bacterial agent of malolactic fermentation (MLF). This species is a Gram-positive, catalase negative, microaerophilic and heterofermentative coccus [14]. With the introduction of molecular techniques, however, a new genus, *Oenococcus,* was described, and *Leuconostoc oenos* was reclassified as *Oenococcus oeni* [10]. Due to its resistance to high ethanol concentrations (<15% v/v) and tolerance of low pH (as low as 2.9), *Oenococcus oeni* is the species of lactic acid bacteria (LAB) most frequently associated with MLF in wine. In this reaction, L-malate is converted to L-lactate and carbon dioxide. MLF promotes the deacidification and microbial stability of wines [16,20,27,40]. However, it can either positively or negatively influence the sensorial profiles of wines, with the overall effects largely dependent on the particular strain involved and on the type of wine being produced [4].

In the last 20 years, molecular biology techniques have provided new information on microbial biodiversity. Yet, it is

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difficult to identify strains within species, especially when microorganisms belonging to a genomically homogeneous species are analyzed. Strains belonging to *O. oeni* are clearly distinguishable from *Leuconostoc* species by chromosomal DNA-DNA hybridization [9,25,26,36,42], 16S and 23S rRNA sequence analysis [11,32,33,36,42], 16S-23S rDNA intergenic spacer region sequencing (ITS-PCR) [23,52] and ribotyping [6,45,46,50,53]. Several studies on genotyping diversity among *O. oeni* strains, carried out using molecular techniques including DNA fingerprinting, restriction endonucleases analysis–pulsed field gel electrophoresis (REA-PFGE) [19,21,22,28,29,36,41,42,45,50], randomly amplified polymorphic DNA-PCR (RAPD-PCR) [2,6,22,24,36–38, 41,50], and differential display PCR (DD-PCR) [22,36,41] suggest that this species is phylogenetically homogeneous, although physiologically diverse. Delaherche et al. [8], based on sequence analyses of nine genes, claimed that *O. oeni* is a single bacterial species displaying genomic variation, which may be correlated to malolactic activity. However, recent studies [39] using multilocus sequence typing (MLST) and physiological characterization have again raised the hypothesis of subspecific divisions within this taxon. Given the taxonomic structure of *O. oeni*, the availability of reliable methods for strain differentiation is crucial for monitoring the survival and contribution of inoculated and autochthonous bacteria and to select individual *O. oeni* strains with desirable organoleptic properties. Since the wine dynamics of *O.oeni* populations are also conditioned [37,38] by the available species and strain diversity (from spontaneous and controlled inoculation) as well as the winemaking conditions (e.g. temperature, wine chemical profile), the identification and typing of MLF-promoting isolates is a reliable approach to assess their ability to dominate the native microbiota and to correlate their dominance/performance with distinct winemaking conditions.

In the present work, 121 *O. oeni* strains were isolated from wines of different winemaking regions of Portugal and identified using a phenotypic and molecular approach. M13-PCR fingerprinting analysis was carried out to evaluate the genetic diversity of this collection of *O. oeni* strains and to search for underlying patterns of regional/geographical strain diversity.

Materials and methods

Bacterial strains. The 121 bacterial isolates of *Oenococcus oeni* used in this work are listed in Table 1. Among them, 100 were isolated from wines, at the end of spontaneous MLF, recovered from four wineries of Dão (Carregal do Sal, Viseu, Mangualde and Mealhada), two wineries of Ribatejo (Dois Portos and Arruda dos Vinhos) and one winery of Alentejo (Reguengos). Additionally, 20 *O. oeni* isolates from Nelas (Dão) and one *O. oeni* isolate from Ourém (Ribatejo), previously isolated and identified [PhD thesis, R. Tenreiro, Univeristy of Lisbon, 1995], were obtained from the

Table 1. *Oenococcus oeni* strains used in this study

Region/Sub-region wine		*Oenococcus oeni* strains
Dão	Nelas	bOg18, bOg20, bOg22, bOg23, bOg27, bOg29, bOg30, bOg31, bOg32, bOg33, bOg34, bOg35, bOg36, bOg39, bOg40, bOg41, bOg42, bOg43, bOg44, bOg45
	Carregal do Sal	DS5
	Silgueiros	ID4, ID5
	Mangualde	ID6, ID38, ID39, ID40, ID42, ID43, ID44, ID45, ID46, ID47, ID48, ID53, ID55, ID56, ID57, ID58, ID62, ID65, ID70
	Mealhada	ID41
Ribatejo	Dois Portos	EVN1, EVN2, ENV7, E169, IO1, IO2, IO24, IO25, IO27, IO30, IO58, IO59, IO60, IO61, IO62, IO63, IO64, IO66, IO75, Agro1, Agro2, Agro3, Agro4, Agro5, Agro6, Agro7, Agro8, Agro9, Agro10, EVN19, EVN22, EVN26
	Ourém	bOg38
	Arruda dos Vinhos	IER1, IER2, IER3
Alentejo	Reguengos	IAL7, IAL8, IAL9, IAL10, IAL11, IAL12, IAL13, IAL14, IAL15, IAL16, IAL17, IAL18, IAL19, IAL20, IAL21, IAL22, IAL23, IAL24, IAL25, IAL26, IAL27, IAL28, IAL29, IAL30, IAL31, IAL33, IAL34, IAL35, IAL36, IAL37, IAL49, IAL50, IAL51, IAL52, IAL54, IAL59, IAL60, IAL61, IAL63, IAL64, IAL66, IAL71

Oenococcus oeni culture collection of the Center of Biodiversity, Functional and Integrative Genomics (BioFIG/FCUL, Lisboa, Portugal). In this study, the type strain *O. oeni* DSMZ 20252^T (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was also included as were seven commercial malolactic starters (Viniflora oenos and Viniflora CH35 from Christian Hansen, Hørsholm, Denmark; GM from Microlife Technics, Sarasota, Florida, USA; Alpha, Beta and VP41 from Proenol, Vila Nova de Gaia, Portugal; PSU-1 from Pennsylvania University, Philadelphia, USA).

Bacteria isolation. The bacteria were isolated by spreading 100 ml of wine samples onto plates with medium promoting the growth of *Leuconostoc oenos* [4] (MLO, tryptone 1%, yeast extract 0.5%, glucose 1%, fructose 0.5%, magnesium sulfate 0.02%, manganese sulphate 0.005%, ammonium citrate 0.35%, Tween 80 0.1%, tomato juice 10% and cysteine 0.05%), adjusted to pH 4.8. Cycloheximide (100 mg /l, Sigma-Aldrich, St. Louis, USA) was added to inhibit the growth of yeasts and molds. The plates were incubated anoxically inside jars containing an Anaerocult C system (Merck, Darmstadt, Germany) at 30ºC for 12 days. Colonies were then selected and further isolated as pure cultures by repeated streaking onto plates containing MTJ medium (70% MRS medium, Merck, Darmstadt, Germany; 30% tomato juice broth, Difco & BD, Franklin Lakes, NJ USA). Bacterial strains were maintained as frozen stocks at –80ºC in MTJ broth media and 20% (v/v) glycerol as cryoprotective agent. Working cultures were cultivated at 30ºC in MTJ broth, until stationary phase. Purity was checked by plating on corresponding agar media and microscopic examination.

Identification of the bacterial strains. Bacterial isolates were first selected on the basis of their genus-specific *Oenococcus* characteristics. Catalase-negative and Gram-positive cocci were screened for the release of CO_2 from glucose based on the production of gas in inverted Durham tubes containing MRS broth [15]. Since this property is shared by other LAB genera, the API 50 CHL system (bioMérieux, Craponne, France) was also used for species identification, according to manufacturer's instructions.

For DNA isolation, the strains were grown in MTJ broth until stationary phase at 30ºC. Cells were recovered by centrifugation and total DNA was obtained using an UltraClean Microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). The DNA concentration was determined spectrophotometrically at 260 nm. Ethidium bromide staining was used to visualize the DNA after electrophoresis through a 1% (w/v) agarose gel (Seakem, Cambrex Bio Science, Rockland Maine, USA). Molecular identification of *O. oeni* strains was performed by 16S rRNA gene amplification and restriction analysis with the enzyme *Fse*I as described by Marques et al. [31]. The results were confirmed by partial sequencing of the 16S rRNA genes of several randomly selected isolates and of the type strain *O. oeni* DSMZ20252^T. 16S rDNA was amplified with the universal primers pA and pH [46] and the amplified fragments were purified using a Concert Rapid PCR purification system (Gibco BRL, Carlsbad, CA, USA). The sequencing reactions were performed using the internal primer 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) at the MWG Biotech sequencing service (Ebersberg, Germany). The BLAST algorithm was used to compare the sequences with those of the U.S National Center for Biotechnology Information GenBank entries [1], and an identification at species level was assumed when at least 97% homology with the 16S rDNA sequence of a known species was determined [43].

M13-PCR fingerprinting. Genomic DNA from all *O. oeni* strains was used as template for PCR fingerprinting using as a primer the M13 minisatellite core sequence (csM13) [17] with the sequence 5′-GAGGGTGGCGGTTCT-3′. Approximately 50 ng of total DNA was subjected to PCR amplification in a reaction mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 200 mM of each deoxyribonucleotide (Invitrogen, Carlsbad, CA, USA), 50 pmol of primer (Invitrogen), and 1 U of Taq DNA polymerase (Invitrogen) in a final volume of 25 ml. The reaction mixtures were subjected to amplification in a thermocycler (Biometra, Goettingen, Germany). PCR cycling conditions consisted of: 94ºC for 5 min followed by 40 cycles of 94ºC for 1 min, 40ºC for 2 min and 72ºC for 2 min, plus one additional cycle at 72ºC for 7 min for chain elongation. PCR profiles were resolved by agarose (1.2% w/v) gel electrophoresis in 0.5× TBE buffer (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA; Invitrogen), at 90 V for 3 h. DNA was visualized under UV light after ethidium bromide staining and the results photographed with Kodak 1D software (Kodak, USA).

Data analysis. The images of the gels were captured using the Kodak electrophoresis documentation software 1D. The images were then saved as TIFF files and exported into the pattern analysis software package BioNumerics version 4.61 (Applied Maths, Kortrijk, Belgium) for processing. To obtain a measure of reproducibility, 12 isolates were randomly selected and analyzed in duplicate. The similarity between each duplicate pair was determined from an analysis based on a dendrogram computed with the Pearson correlation coefficient and the unweighted pair group method with arithmetic average (UPGMA) as the agglomerative clustering [47]. The reproducibility value was determined as the average value for all pairs of duplicates. Strain relationships, based on the molecular characters as determined from the fingerprints, were analyzed by hierarchical numerical methods with Pearson correlation similarity and UPGMA clustering. A cut-off value of 85% similarity was used to distinguish the clusters. The intraregional genomic diversity of *O. oeni* was evaluated with the indexes of Simpson [18] and Shannon [51]. The Simpson index (*D*) measures the probability of two non-related strains, taken from the tested population, belonging to two different genomic types and is based on the number of types and isolates for each type. The Shannon index (*J'*) is an evenness measure, expressing the observed diversity as the proportion of the possible maximum diversity and reflecting the homogeneity/heterogeneity of the distribution of isolates among the genomic types.

Results and Discussion

Isolation and identification of the strains. From 81 wines (23 Dão wines, 24 Ribatejo wines and 34 Alentejo wines), a culture collection of 100 bacterial isolates (23 from Dão, 35 from Ribatejo and 42 from Alentejo) was obtained. A primary classification was performed based on cell morphology and cellular arrangement, Gram staining, catalase activity, and CO_2 production from glucose. All isolates were Gram-positive, catalase negative, had similar cell arrangements (single, pairs and long chains), and were heterofermentative. The isolates showed the same fermentation pattern in API 50 CHL galleries, producing acid only from arabinose, esculin, fructose, galactose, glucose, and xylose. As six non-matching tests with the most closely related taxon (*Lactobacillus brevis*) were obtained, no acceptable phenotypic identification was possible using the API database. These results further reinforce the low reliability of this system as an identification tool for wine LAB, especially *O. oeni*, as described by others [34, and PhD thesis, R. Tenreiro 1995].

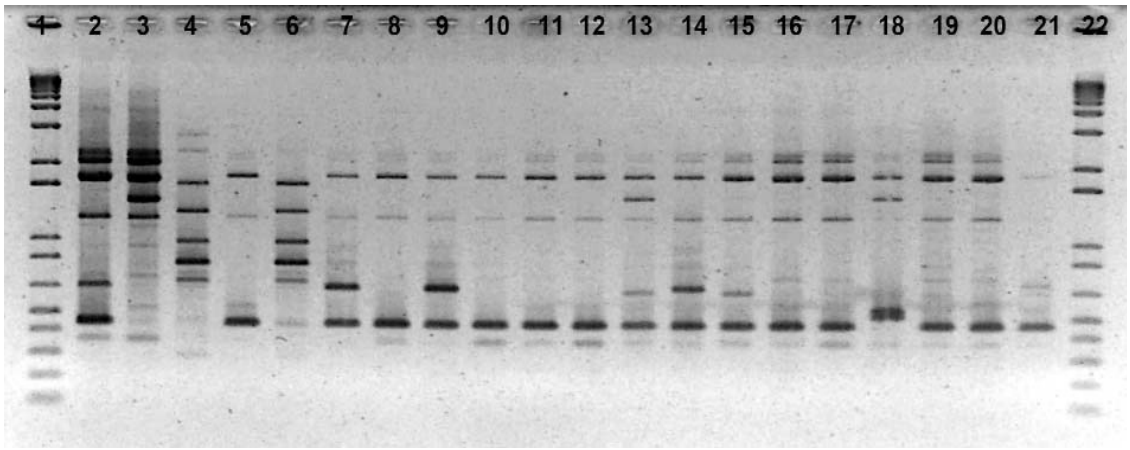


Fig. 1. Representative M13-PCR profiles of several strains of *Oenococcus oeni*. Lanes 1 and 22: molecular ladder 1 kb plus (Invitrogen); lanes 2–21: *O. oeni* isolates from wines of different winemaking regions in Portugal.

However, the assays used for the primary classification offer a practical screening strategy and allowed us to conclude that the bacterial isolates belonged to a group of heterofermentative cocci LAB.

The bacterial isolates were identified as *O. oeni* using the molecular methodology described by Marques et al. [31]. This method is based on 16S rRNA gene amplification with universal primers followed by restriction enzyme analysis with the endonuclease *Fse*I, generating two fragments of 326 and 1233 bp. These results were confirmed based on the partial 16S rDNA sequence of some isolates (10%), randomly selected, and that of the type strain *O. oeni* DSMZ20252^T. The DNA sequences were analyzed and compared using the BLAST network service (NCBI). The resulting fragments were approximately 98% similar to the 16S rRNA gene isolated from an *O. oeni* strain (GenBank accession number X95980), confirming that the isolated strains belonged to *O. oeni* species (data not shown).

M13-PCR fingerprinting. The intraspecific diversity of our culture collection of 121 *O. oeni* strains obtained from three winemaking regions throughout Portugal was evaluated by M13-PCR fingerprinting analysis. The primer csM13 provided suitable fingerprints, with well defined amplification patterns (Fig. 1).

The reproducibility of the fingerprints with primer csM13, estimated by the similarity average value for all pairs of duplicates, was 96 ± 0.4%. The DNA fingerprinting patterns were analyzed on BioNumerics software (v4.61, Applied Maths) and the genetic similarity between the 121 *O. oeni* strains was displayed in the form of a dendrogram, depicted in Fig. 2.

The cophenetic correlation coefficient was 0.93, which demonstrates the faithfulness of a dendrogram in preserving the pairwise distances between the original unmodeled data points. Although a value of 1.0 means that the concordance (as a linear relation) between the input data and the tree is theoretically perfect, in practice the relationship is unlikely to be totally linear. Romesburg [Cluster Analysis for Researchers. Wadsworth, Inc., USA, 1984] suggested that a cophenetic correlation of 0.8 or above indicates that the dendrogram does not greatly distort the original structure in the input data. However, the cophenetic correlation coefficient is not always a very reliable measure of the distortion due to a hierarchical model [12].

At a similarity level of 85%, the M13-PCR fingerprinting analysis organized the *O. oeni* strains in 49 genomic groups (27 different genomic clusters, represented by two or more isolates and 22 single-member genomic clusters). Six major genomic clusters (I–VI) were also defined, based on the overall hierarchical relationships, with distinctive composition in terms of the regional origin of the isolates. *O. oeni* strains from the Dão region were distributed into 19 genomic groups, including seven unique profiles as single-member clusters. Strains from the Ribatejo region were grouped in 22 genomic groups, with ten of them as single-member clusters, while those from the Alentejo region belonged to 14 genomic clusters, with five single-member clusters. Although nine out

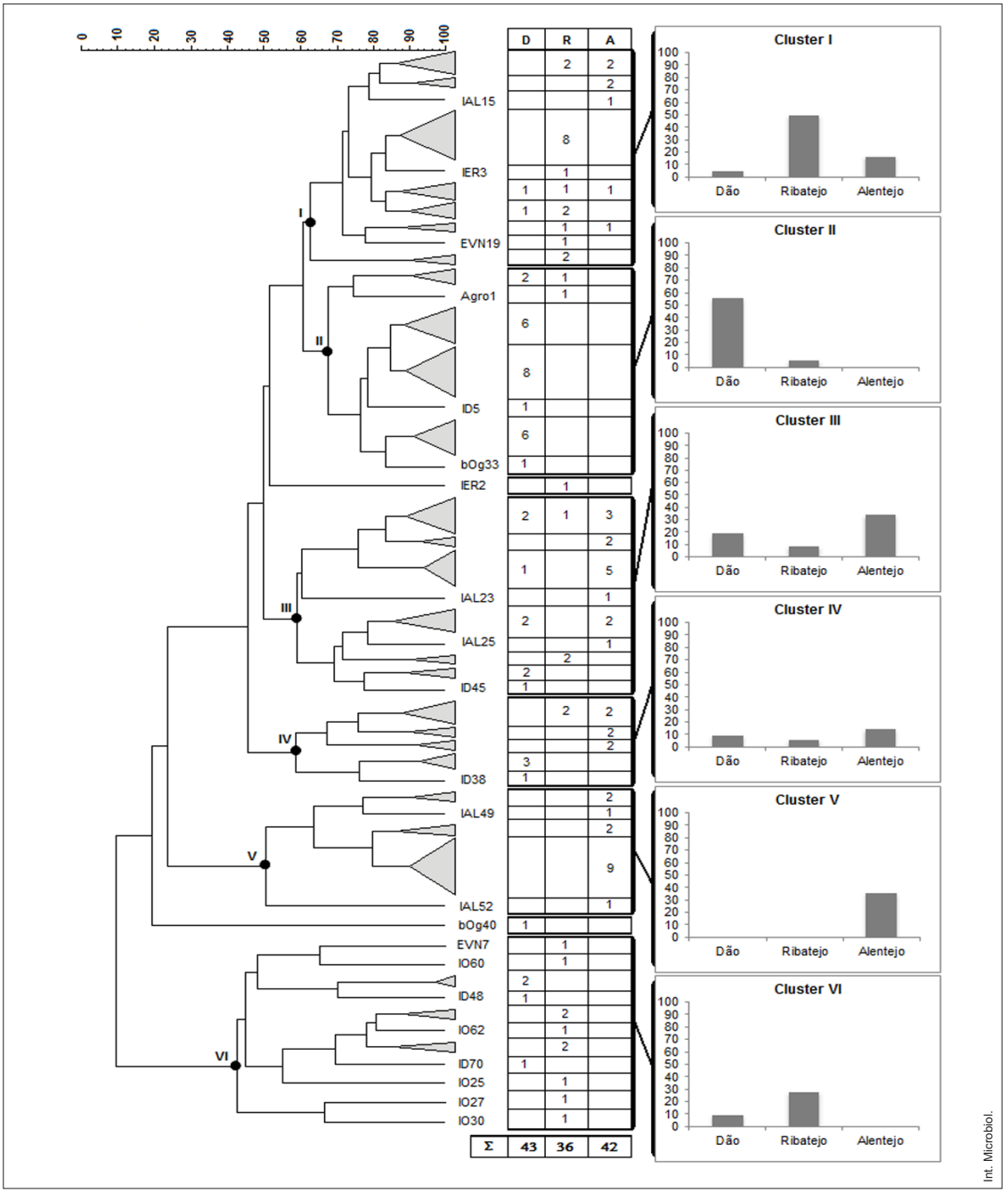


Fig. 2. Dendrogram of the 121 *Oenococcus oeni* isolates from different winemaking regions in Portugal based on the M13-PCR fingerprint analysis (Pearson correlation coefficient and UPGMA clustering). Alphabetic letters indicate the genomic groups of strains defined at an 85% similarity. The number of isolates from each region is displayed. (D: Dão; R: Ribatejo; A: Alentejo), as is the relative distribution of strains in each major cluster I-VI (as a percentage of the total number per region).

Table 2. *Oenococcus oeni* diversity indexes for the isolates in winemaking regions

	Diversity index*		
Winemaking region	% Types	*D*	*J'*
Dão	47 (20/43)	0.93	0.89
Ribatejo	50 (18/36)	0.92	0.91
Alentejo	43 (18/42)	0.93	0.92

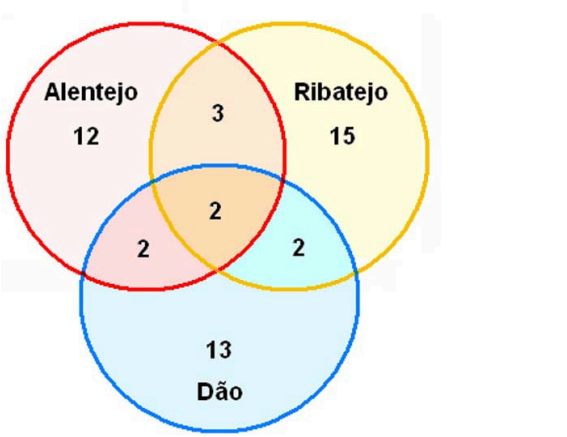
**D*: Simpson diversity index; *J'*: Shannon diversity index; % Types: (number of types/number of isolates)×100, in each winemaking region.

of the 27 genomic clusters (A, D, E, F, H, L, N, O, and R) comprised a mixture of *O. oeni* isolates from more than one region (9 isolates from Dão, 10 from Ribatejo, and 16 from Alentejo), the remaining 18 genomic clusters were formed only by isolates from the same region (6 from Dão, 5 from Ribatejo, and 7 from Alentejo), pointing to a regional partitioning of the genomic diversity in this species. *O. oeni* isolates from the same wine were distributed by different clusters, which indicated the presence of different types of *O. oeni* strains in the same wine.

Seven commercial malolactic starters (VP41, Alpha, Beta, Viniflora oenos, Viniflora CH35, GM, and PSU-1) and the *O. oeni* type strain (DSMZ 20252^T) were also submitted to fingerprint analysis. For each starter, a unique and discriminative DNA fingerprint was obtained, with the exception of the starters Viniflora oenos and Viniflora CH35, which were grouped in the same genomic cluster (data not shown). Each of these commercial *O. oeni* strains has different winemaking origins.

Shannon-Weaver and Simpson diversity indexes were applied to assess the intra-regional genomic diversity of *O. oeni* strains from the different winemaking regions of Portugal (Table 2). Both the percentage of types and the values of the Simpson and Shannon-Weaver diversity indexes, obtained with M13-PCR fingerprinting, were closely similar and high enough for each winemaking region so as to confirm the high genomic diversity of *O. oeni*, as previously determined by MLST, macrorestriction, and physiological characterization [35,39,45].

Evaluation of regional distribution of *Oenococcus oeni* genomic groups. Among the 49 genomic groups defined by M13-PCR fingerprinting analysis (Fig. 2), 40 were unique to a particular winemaking region. Seven genomic groups (A, E, F, H, N, O, and R) were shared by two regions each, while the remaining two (D and L) were



M13-PCR genomics groups	Dão	Ribatejo	Alentejo	Total
Unique	13	15	12	40
Shared	6	7	7	9
Total	19	22	19	49

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Fig. 3. Regional distribution of the 49 M13-PCR genomic groups of *Oenococcus oeni* isolates from Portuguese wines of different winemaking regions.

the only ones that included isolates from the three regions. When the uniqueness/commonness ratio of genomic groups was analyzed for each winemaking region (Fig. 3), a 2:1 proportion was found between specific genomic profiles unique to that region and genomic profiles shared with at least another region. This pattern of geographically associated diversity is also obvious from the composition of the six major genomic clusters (I–VI; Fig. 2) in terms of the regional origin of the isolates. Overall, these data point to a global partitioning of the genomic diversity of *O. oeni* according to the geographical origin of the isolates and to the occurrence either of an alopatric or ecological speciation process in this wine species. Similar conclusions have been reached in other bacterial groups subjected to highly selective or heterogeneous environments [49].

During the last several years, the diversity of *O. oeni* strains within and around wineries has been extensively examined. The results obtained from the application of different techniques, such as studies of the patterns of total soluble cell proteins [9], 16S and 23S sequence analyses [32], RAPD-PCR [53] and DD-PCR [22], suggest that *O. oeni* is a homogeneous species. More recently, de las Rivas et al. [7] submitted five genes (*gyr*B, *ddl*, *mle*A, *pgm*, and *rec*P) to MLST in order to evaluate the allelic diversity and population structures of various oenococcal isolates. This analysis was able to completely differentiate 18 strains, suggesting a higher level of genetic heterogeneity among oenococcal isolates. These authors argued that the high level of diversity in *O. oeni* is an example of a panmictic genetic population, in which the high frequency of recombination among constituents results in the randomization of sequences and the generation of linkage equilibrium. Marcobal et al. [20] showed that high mutation rates in *O. oeni* explain some of the discordant observations reported for this species. They suggested that the lack of *mut*S and *mut*L in *O. oeni*, combined with the high mutation rate, accounts for the high allelic diversity among strains, as seen from the MLST data.

In oenology, biodiversity is strictly correlated to habitat. Consequently, it is conditioned by selective factors that inhibit or favor the presence not only of one species over the other but also of a strain or biotype. The present study aimed to differentiate *O. oeni* isolates from different winemaking regions of Portugal and to reveal the underlying patterns of regional/geographical strain diversity. Our results confirm the predominance of *O. oeni* species in the hostile conditions prevailing in wine and the high adaptation capacity of the various strains in the winery environment [53]. M13-PCR fingerprinting allowed the genomic discrimination of *O. oeni* while a cluster analysis of M13-PCR patterns revealed a correlation between strain distribution and geographical area of origin. This approach can be useful in following the evolution of *O. oeni* populations during malolactic fermentation in wine and in assessments of the *O. oeni* ecology in wine.

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3.3 Evaluation of oenological characteristics of Portuguese autochthonous *Oenococcus oeni* strains for the selection of malolactic starters

Evaluation of oenological characteristics of Portuguese autochthonous *Oenococcus oeni* strains for the selection of malolactic starters

Summary

In order to select autochthonous *O. oeni* strains from different winemaking regions of Portugal for malolactic starters to be used in wine industry, a set of 129 *O. oeni* strains was used to assess malolactic and β-glucosidase activities, production of biogenic amines and ethyl carbamate precursors. A set of 51 *O. oeni* strains was used to study the growth behaviour under different conditions. Most of the *O. oeni* strains revealed β-glucosidase activity and high levels of malolactic activity. No production of histamine, tyramine, phenylethylamine, isoamylamine and agmatine was detected and only up to 28 *O. oeni* strains were detected as capable to produce putrescine and/or cadaverine. The production of low amounts of ethyl carbamate precursors from arginine degradation appears to be a common ability. Our results emphasize the importance of the characterization of *O. oeni* strains regarding the production of enzymes and undesirable compounds as criteria for the selection of malolactic starters. By applying multivariate statistics to data obtained from growth behaviour of Portuguese strains and commercial malolactic starters under different conditions, a strain from each winemaking region of Portugal could be selected as the most suitable regional O. *oeni* strain for malolactic fermentation.

Introduction

In winemaking one of the most difficult steps to control is the malolactic fermentation (MLF), which normally occurs after completion of the alcoholic fermentation. MLF is conducted by lactic acid bacteria (LAB), mainly *Oenococcus oeni*. The activity of these bacteria decreases wine total acidity,

improves microbiological stability and enhances its organoleptic properties (Zapparoli *et al*., 1998; Lounvaud-Funel, 1999). Relying on the indigenous bacterial microflora to complete a timely and desirable MLF can be counter-productive. When desirable malolactic bacteria are established in a winery, the onset of the MLF may take several months and may occur in some barrels and tanks but not in others. For this reason, induction of MLF by the use of selected *O. oeni* starter cultures is becoming the preferred option. Nevertheless, some strains are considered more desirable for inducing MLF than others due to their different and possibly unique malolactic activity and growth characteristics (Lafon-Lafourcad *et al*., 1983; Davis *et al*., 1988; Britz and Tracey, 1990; Davis *et al*., 1995). Historically, the selection criteria for malolactic starters have been based on growth at low pH, resistance to sulphur dioxide (SO_2) and ethanol, survival in wine and high levels of malolactic enzyme activity (Liu and Gallander, 1983; Henick-Kling *et al*., 1989; Wibowo *et al*., 1998). Lack of or low capacity to produce biogenic amines (BA) and ethyl carbamate precursors, as well as high levels of glycosidase activity have also been included in the selection criteria of malolactic starters activity (Liu and Gallander, 1983; Henick-Kling *et al*., 1989; Wibowo *et al*., 1998). BA can cause healthy disorders if large amounts are ingested or if the natural detoxification process is inhibited or genetically deficient (Bauza *et al*., 1995). The study of the occurrence of BA in wine is particularly important as ethanol can enhance their toxic effects on human metabolism by inhibiting the amine oxidases responsible for their catabolism (Straub *et al*., 1995).

Arginine is the most abundant amino acid in wine. One major concern in arginine and citrulline metabolism by wine LAB is their association with the formation of ethyl carbamate (EC), since EC can induce some kinds of tumours in animals and humans (Arena *et al*., 1999; Moreno-Arribas *et al*., 2000). For this reason, the wine industry is interested in reducing EC levels in their products (Field and Lang, 1988; Schlatter and Lutz, 1990; Zimmerli and Schlatter, 1991).

On another hand, β-glucosidase activity is involved in the hydrolysis of several important compounds for the development of varietal wine flavour. Typically, the target glycosides are β-D-glucose and diglycoside conjugates. Volatile aglycones present as monoglucosides are liberated via β-D-glucosidase activity (Günata *et al*., 1988; D'Incecco *et al*., 2004).

Selecting the strains of *O. oeni* with the best performance and that are most interesting for winemaking is a complex and challenging task. Therefore, the main objective of this study was the selection of *O. oeni* strains to be used as MLF starters on oenological industry. To achieve this aim, *O. oeni* strains previously isolated from Portuguese wines were characterized in terms of malolactic and β-glucosidase activity and production of biogenic amines and ethyl carbamate precursors. A set of 51 *O. oeni* strains were selected and tested for their potential to grow under different conditions, comparing favourable and harsh ones.

Material and Methods

Microorganisms

A total of 152 LAB strains (149 *O. oeni* strains, one *Lactobacillus buchneri* and two *L. brevis*) were used in this study. One hundred and twenty one *O. oeni* strains were isolated from Portuguese wines from winemaking region of Dão, Ribatejo and Alentejo (Marques *et al*., 2011). Twenty additional *O. oeni* and two *L. brevis* (AI249 and AI445) were isolated from wines of Douro region (Inês, 2007). Seven commercial malolactic starters (Alpha, Beta and VP41 from Proenol, Portugal; Viniflora oenos and Viniflora CH35 from Christian Hansen, Denmark; GM from Microlife, USA; and PSU-1 from California, USA), the *O. oeni* type strain (DSMZ 20252^T) and one *L. buchneri* reference strain (DSMZ 5987), were also included in this study.

Inoculum preparation

Strains were grown at 30ºC until the end of exponential phase in MTJ medium (70% MRS, Merck, Darmstadt, Germany plus 30% Tomato Juice Broth, Difco™ & BD™, Franklin Lakes, NJ USA) at pH 4.8. The cells were harvested by centrifugation washed with sterile distilled water and ressuspended in sterile distilled water to an OD 600 nm of approximately 0.9. Unless otherwise stated, this bacterial suspension was used to inoculate the experimental media at a rate of 2% (v/v).

Determination of malolactic activity

The strains were inoculated in MTJ medium supplied with L(-)-malic acid (5 g/L) and incubated at 30ºC. The amount of L-lactic acid production was measured using the enzymatic test kit of Boehringer Mannheim (Germany) when the cells were at the end of exponential phase. To evaluate concentration of L(-)-lactic acid, a standard curve was made using known concentrations of L(-)-lactic acid (1, 3 and 5 g/L). The assays were performed according to the supplier instructions. To evaluate reproducibility, duplicates were used for a random sample of 10% of the *O. oeni* strains.

Determination of β-glucosidase activity

β-glucosidase activity was assayed as described by Rosi *et al*. (1994) and also by measuring the amount of p-nitrophenol (pNP) liberated from p-nitrophenyl-β-D-glucoside (pNPG) as substrate using a colorimetric method. The cellular suspension of each bacterial strain was inoculated in MTJ medium and incubated at 30ºC till the end of exponential phase. The cultures were centrifuged (10000 *g,* 10 min, 4ºC) and the culture supernatant fluid was assayed for enzymatic activity. A tube containing uninoculated media was used as the control. The assay was performed in a microplate of 96 wells using 25 μL of pNPG (5 mM), 25 μL of acetate buffer and 50 μL of sample per well. The microplate was agitated and incubated at 50ºC during 30 minutes and

subsequently 100 μL of glycine buffer (400 mM, pH 10.8) was added to stop the reaction. The liberated pNP was measured by spectrophotometry at 430 nm and compared with a series of standards prepared with pNP at concentrations ranging from 0 to 500 μM. Controls without cells were treated in the same way as samples and included in the same 96-well plate. A unit of activity was defined as nmoles of pNP liberated per millilitre per hour. The *O. oeni* AI 359 (Inês, 2007) was used as positive control. The assays were performed in duplicated for 10% of the *O. oeni* strains, to assess reproducibility.

Determination of biogenic amine-producing ability

The biogenic amine-producing ability was evaluated. Amino acid decarboxylation was tested by inoculating each LAB strain in biogenic amines production medium (BAPM) pH 5.5, with each amino acid (3 g/L) to be tested (histidine, tyrosine, ornithine, lysine, phenylalanine, isoleucine or arginine) (Landete *et al*., 2005a) and purple bromocresol as pH indicator. The bacterial strains were incubated at 30ºC until stationary phase. The analysis of biogenic amines (histamine, tyramine, putrescine, cadaverine, phenylethylamine, isoamylamine and agmatine) was performed by reverse-phase high-pressure liquid chromatography (RP-HPLC) according to the method described by Vidal-Carou *et al*., (2003). Briefly, the liquid chromatograph is composed by a Waters 510 Pump, a Waters 715; an Auto-sampler, a Waters Temperature Control Module and a Waters 474 Fluorescence Detector (Waters Chromatography, Milford, MA, USA). Data acquisition was accomplished with the Millenium32 version 3.05.01, 1998 system (Waters Chromatography). The chromatographic separations were carried out using a Nova-Pack C18, 4 μm (150 x 3.9 mm) (Waters Chromatography). The reference strain *L. buchneri* DSMZ 5987 and *L. brevis* AI249 and AI445 (Inês, 2007) were used as positive controls. Reproducibility was assessed by duplicate assays for 10% of the *O. oeni* strains randomly selected.

Determination of ethyl carbamate precursors

The detection of citrulline and ornithine production from arginine degradation via arginine deiminase (ADI) pathway was performed using the colorimetric methods adapted from Sugawara *et al*., (1998) and Chinard, (1952), respectively, in microplates of 96 wells. The bacterial cell suspension was inoculated into the MTJ medium supplied with arginine or citrulline at a concentration of 1 g/L. These cultures were incubated at 30ºC and samples were taken at the stationary phase.

Ammonia was detected qualitatively with Nessler's reagent as described by Pilone *et al*. (1991). The type strain *O. oeni* DSMZ 20252^{T} was used as positive control. Duplicate assays were performed with a random sample of 10% of the *O. oeni* strains to evaluate reproducibility.

Molecular analysis

The molecular analysis of our culture collection of LAB strains was performed searching for: malolactic enzyme gene (*mle*A), histidine, tyrosine and ornithine decarboxylase genes (*hdc*A, *tyr*DC, *odc*, respectively), arginine deiminase operon (*arc*AC, *arc*D1 and *arc*D2) and β-glucosidase gene (*bgl*H). Genomic DNA from each bacterial strain was isolated using UltraClean^{TM} Microbial DNA Isolation Kit (MO-BIO Laboratories, Carlsbad, CA, USA), as described by the supplier. The amplification of *mle*A gene, *hdc*A, *tyr*DC and *odc* genes, *arc*AC, *arc*D1 and *arc*D2 genes and *bgl*H gene was performed by polymerase chain reaction (PCR). Amplification primers, positive controls and size of amplicons of each gene are described in Table 1. PCR reactions and conditions are described in Table 2. The PCR products were observed by gel electrophoresis on a 1% agarose gel using Tris-borate EDTA buffer. After staining with ethidium bromide, the DNA bands were visualized under UV illumination and gel image was captured using a KODAK 1D system (version 3.6) (Kodak, USA). Confirmation of identity of amplified products was performed by

sequencing amplicons obtained from positive control strains and from 10% randomly selected LAB strains.

Table 1 – Primers, amplicon size and positive control used in the amplification of the genes under study.

Gene	Primer	5′ → 3′ sequence	Amplicon size (bp)	Positive control strain
*hdc*A[1]	JV16HC	AGATGGTATTGTTTCTTATG	367	*L. buchneri* DSMZ5987
	JV17HC	AGACCATACACCATAACCTT		
*tyr*DC[2]	P2-for	GAYATIATIGGIATIGGIYTIGAYCARG	924	*L. brevis* AI249; AI445
	P1-rev	CCRTARTCIGGIATIGCRAARTCIGTRTG		
odc[3]	3	GTNTTYAAYGCNGAYAARACNTAYTTYGT	1446	*O. oeni* RM83
	16	TACRCARAATACTCCNGGNGGRTANGG		
arc[5] *arc*AC	RTA5’	CAAGTGAGTTGTCTCGTG	1200	
	RTA3’	GATAAGATAGCATTGCCAC		
*arc*D1	B11	ATGTTTTCCTTTTTAATTACTGC	236	*O. oeni* DSMZ20252[T]
	B12bis	TAAAAGCATTAGTAAAAG		
*arc*D2	B5	TGTTCTCTCGATTAATTTGG	588	
	B6	AGCACATAAATTGCAAGC		
bgl[6]	GLUF	TATCATCATTATAMAGAWGA	1200	*O. oeni* AI359
	GLUR	TCGACATAAATAAAICCRTA		
*mle*A[6]	OO1	GTGCCGCTTTTTTGGATATTA	430	*O. oeni* DSMZ20252[T]
	OO2	AGCAATTTTATCTTTATAGCT		

1 - Le Jeune *et al*., 1995; 2 - Lucas and Lonvaud-Funel, 2002; 3 - Marcobal *et al*., 2004; 4 - Divol *et al*., 2003; 5 - Ohrmund and Elrod, 2002; 6 - Divol *et al*., 2003.

Table 2 - PCR conditions used to detect each gene under study.

Gene	Primer (μM)	PCR			Cycle number
		Step 1	Step 2	Step 3	
*hdc*A[1]	1.0	95ºC, 30 s	40ºC, 30 s	72ºC, 2 min	40
*tyr*DC[2]	0.5	95ºC, 1 min	52ºC, 1 min	72ºC, 1 min	35
odc[3]	0.5	95ºC, 1 min	48ºC, 1 min	72ºC, 1 min	35
*arc*AC[4]					
*arc*D1[4]	0.5	95ºC, 1 min	43ºC, 1 min	72ºC, 1 min	35
*arc*D2[4]					
bgl[b]	1.5	95ºC, 1 min	48ºC, 1min	72ºC, 1 min	35
*mle*A[b]	1.0	95ºC, 1 min	50ºC, 1 min	72ºC, 1 min	35

1 - Le Jeune *et al*., 1995; 2 - Lucas and Lonvaud-Funel, 2002; 3 - Marcobal *et al*., 2004; 4 - Divol *et al*., 2003; 5 - Ohrmund and Elrod, 2002; 6 - Divol *et al*., 2003.

Growth assays

O. oeni strains were pre-inoculated in MRS (Merck) supplied with 0.5% malic acid pH5.5 and incubated at 30ºC until the end of exponential phase. The cells were recovered by centrifugation and washed twice with sterile 0.85% NaCl. The cellular suspension was inoculated in each culture medium at identical optical density (0.2). The assayed conditions are described in Table 3. The bacterial strains were grown in a "synthetic wine" (A to J) (Liu *et al*., 1995) (containing tartaric acid, malic acid, ethanol, vitamins, organic acids, purines and pyrimidines bases) and also in MRS (Merck) supplied with 0.5% of malic acid pH 5.5 at two different temperatures (K and L). The optical density was measured at 600 nm every two days, during 18 days. All the assays were performed in duplicate.

Table 3 - Composition and codes of the different synthetic wine conditions (A to J) and culture medium (K to L) and growth temperatures.

Growth conditions	A	B	C	D	E	F	G	H	I	J	K	L
pH	3.5						3.2	3.8	3.5		5.5	
Ethanol (%)	11				8	14	11				0	
Malic acid (%)	0.5								0.2	0.5		
SO_2 (mg/L)	0	10	20	30	0							
Temperature (ºC)	25									20	25	20

Data analysis

To analyse the absorbance versus time data, data were exported and organized into spread sheets and a trapezoidal approximation was used to determine the area under the absorbance versus time curve for each sample. The analysis of relative trapezoidal area of each curve was performed according Guckert *et al*. (1996). The matrix of optical density values (ODi) was designed in MSEXCEL and converted, by using the initial OD_i value (OD_{i0}), in absolute values of relative optical density (ODr_i) calculated according to the formula:

$$ODr_i = \left| \frac{OD_i}{OD_{i0}} - 1 \right|$$

The absolute values of the relative optical density define a curve that established the variation of relative optical density during the experimental time. For each curve the relative trapezoidal area (RTA) was calculated, using the formula:

$$RTA = \frac{\Delta t}{2}\left[ODr_0 + ODr_n + 2\sum_{i=1}^{n-1} ODr_i \right]$$

where t represents the reading interval and ODr_0, ODr_n and ODr_i are the absolute values of the relative optical density corresponding to initial, final and time t_i measures, respectively. After direct comparison of the RTA values, the obtained results were also analysed by hierarchical (cluster analysis) and non-hierarchical methods (principal components analysis) methods using the NTSYSpc software (ver. 2.2; Exeter Software, USA) software.

Results

Malolactic and β-glucosidase activities

The malolactic activity was evaluated as the amount of L(-)-lactic acid present in the culture medium. Of the 129 *O. oeni* strains tested, 124 (95%) were able to produce more than 4 g/L of lactic acid and only 5 (4%) strains produce less than 4 g/L of lactic acid.

LAB strains with β-glucosidase hydrolyse the substrate arbutine and a dark brown colour develops in the agar plate. This method allowed the detection of 102 out of 121 (80%) *O. oeni* strains able to produce β-glucosidase. However, the colorimetric method revealed β-glucosidase enzyme activity in all (121) *O. oeni* strains. Eighty percent (97/121) of *O. oeni* strains showed less than 250 units of β-glucosidase activity, 16% (19/121) 250 to 1000 units and only four percent (5/121) above 1000 units. As levels of β-glucosidase activity of *O. oeni* strains from different winemaking regions overlapped (data not shown), the hypothesis of a geographical association with this character was ruled out.

Biogenic amines production

The amino acid-decarboxylase capacity of lactic acid bacteria (121 *O. oeni* strains previously isolated by Marques *et al*. (2011), seven commercial malolactic starters, reference strains *L. buchneri* DSMZ 5987, *L. brevis* AI249 and AI445, and *O. oeni* DSMZ 20252^T) was screened using the method described by Landete *et al*. (2005a). Biogenic amine-positive reactions were recorded when a purple colour formed in the decarboxylase medium as a result of lactic acid bacteria metabolism. From the 121 tested *O. oeni* strains, only 28 showed ability to produce putrescine. *L. buchneri* DSMZ5987 showed ability to produce histamine and the two *L. brevis* strains showed ability to produce tyramine. As the decarboxylase medium assay may result in numerous false negative or positive responses (Moreno-Arribas *et al*., 2003), the amine-forming capacity of all lactic acid bacteria was then quantified by RP-HPLC. This analysis confirmed the ability of the 28 *O. oeni* strains to produce putrescine from ornithine and also showed the ability of 26 *O. oeni* strains to produce cadaverine from lysine. Twenty three (82%) of the putrescine producers showed ability to produce amounts above 1000 mg/L, three (11%) produced amounts between 500 and 1000 mg/L and two (7%) produced amounts below 500 mg/L. The cadaverine producers only showed ability to produce amounts below 20 mg/L. All the cadaverine producers also revealed ability to produce putrescine. The 129 *O. oeni* strains, in these growth conditions, did not have the capacity to produce histamine, tyramine, phenylethylamine, isoamylamine and agmatine. The seven commercial malolactic starters and the *O. oeni* type strain DSMZ 20252^T in these growth conditions did not show ability to produce biogenic amines. *L. buchneri* DSMZ 5987 only produced histamine and the two *L. brevis* strains isolated from Douro wines only produced tyramine.

Ethyl carbamate precursor production

A set of 129 *O. oeni* strains (121 strains previously isolated by Marques *et al*. (2011), seven commercial malolactic starters and *O. oeni* DSMZ20252^T) were tested for their ability to produce ethyl carbamate precursors (citrulline and carbamyl phosphate) via ADI pathway. The ability to produce EC precursors was evaluated by the quantitative detection of citrulline and ornithine using colorimetric methods. Arginine is converted into citrulline than converted into ornithine and carbamyl phosphate. If the amount of ornithine produced during the ADI pathway is determined, it is possible to evaluate if that strain produces carbamyl phosphate and estimate its quantity. The ammonia assay allowed to detected 100 (83%) *O. oeni* strains able to degrade arginine into ammonia via ADI pathway. The detection of citrulline and ornithine production allowed confirming the results obtained with ammonia assay, but also showed that more 18 (15%) *O. oeni* strains also have the capability to degraded arginine and only 3 (2%) *O. oeni* strains did not have the ability to consume arginine. Seventy five percent (89/118) of *O. oeni* strains produced citrulline below 500 μM and only 8% (9/118) produced amounts above 1000 μM. Seventy one percent (84/118) of *O. oeni* strains produced ornithine below 500 μM and only 3% (4/118) produced amounts above 1000 μM. *O. oeni* strains from Dão and Alentejo produced fewer amounts of citrulline and ornithine than strains from Ribatejo (data not shown). From the seven commercial malolactic starters, four (Alpha, VP41, Viniflora oenos and PSU-1) were able to degrade arginine, via ADI pathway, but in very low amounts (data not show).

Molecular characterization

PCR amplification was used to screen histidine, tyrosine and ornithine decarboxylase genes (*hdc*A, *tyr*DC and *odc*, respectively), arginine deiminase gene cluster (*arc*AC, *arc*D1 and *arc*D2), β-glucosidase gene (*bgl*H) and malolactic enzyme gene (*mle*A) in our LAB culture collection. The *hdc*A gene was only detected in the reference strain *L. buchneri* DSMZ 5987 (positive

control). The *tyr*DC gene was only detected on both *L. brevis* strains isolated from Douro wine that had shown ability to produce tyramine from tyrosine on HPLC assay. The *odc* gene was detected in the 28 *O. oeni* strains able to decarboxylate ornithine into putrescine. The *arc* gene cluster was detected in all *O. oeni* strains able to degrade arginine. *bgl*H gene was detected in all *O. oeni* strains. All the amplicons showed the expected size for each gene (Table 1) and identity was confirmed by sequencing of a random sample.

Growth assays

Based on molecular typing analysis by M13-PCR fingerprinting (Marques *et al*., 2011) and the enzymatic and genetic analysis of desirable oenological characteristics of our culture collection of 121 *O. oeni* strains, a set of 26 *O. oeni* strains was selected for growth studies. Nineteen *O. oeni* strains from Douro wines, previously characterized in terms of enzymes with oenological relevance (Inês, 2007) and six commercial malolactic starters, were also added to this set of strains.

All the 51 *O. oeni* strains were able to grow at low temperature (20ºC) and in the presence of ethanol (8 and 14%) and low pH (3.5 and 3.8). But only 15 were able to grow in the presence of 10 mg/L SO_2, six in the presence of 20 mg/L SO_2 and four in the presence of 30 mg/L SO_2. Only one *O. oeni* strain was not able to grow at pH 3.2. The *O. oeni* strains, from each winemaking region of Portugal, that grew better in the presence of SO_2 were AI202 (Douro region), ID58 (Dão region) and bOg18 (Ribatejo region) (Figure 1). None strain from Alentejo was able to grow in the presence of SO_2. The *O. oeni* strains from each winemaking region that grew better at low pH and in the presence of ethanol were AI202, ID58, Agro1 (Ribatejo region) and IAL33 (Alentejo region) (Figure 1).

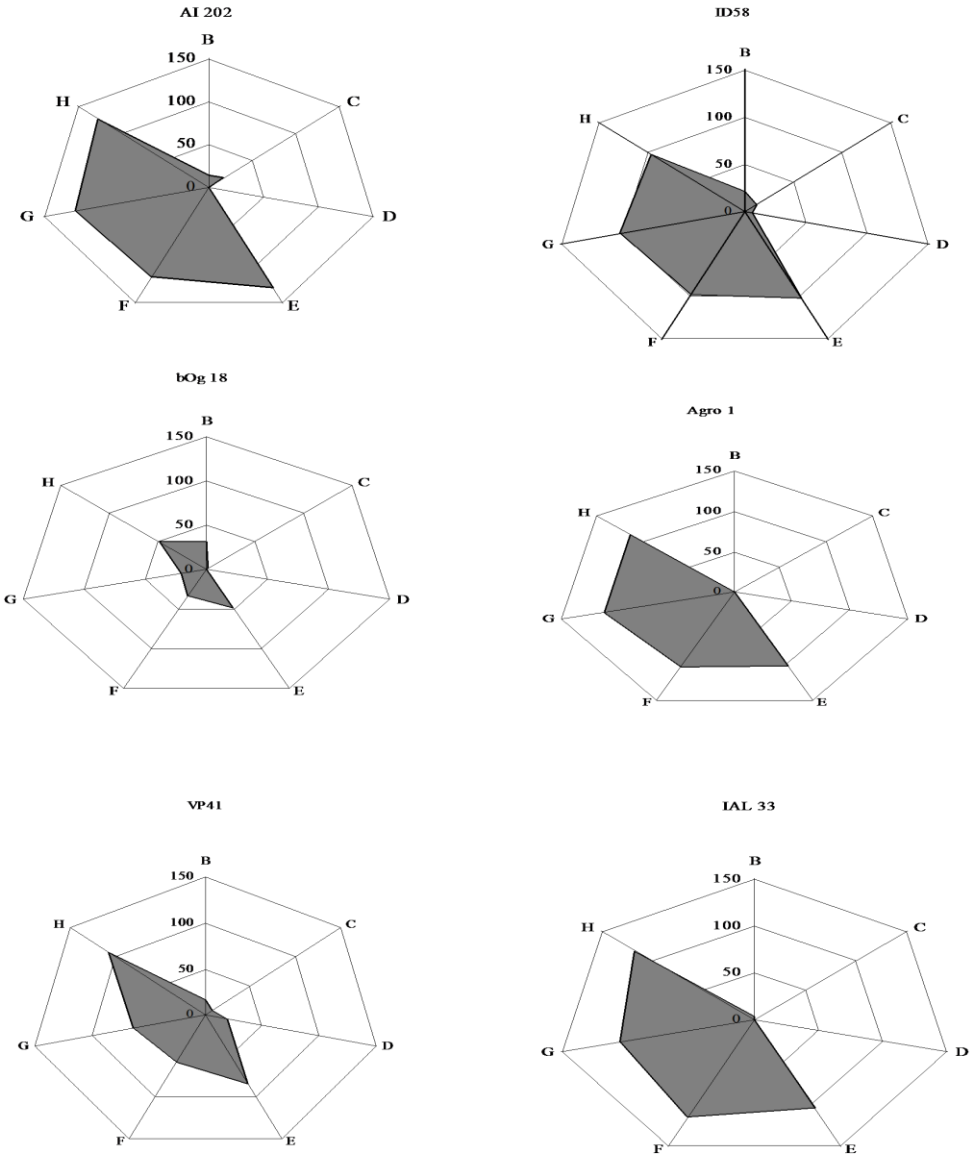

Figure 1 - Growth behaviour in response SO_2, ethanol and pH of five *O. oeni* strains selected from winemaking regions of Portugal and starter VP41. Scale refers to value of trapezoidal area under growth curve. B: 10 mg/L SO_2; C: 20 mg/L SO_2; D: 30 mg/L SO_2; E: 8% ethanol; F: 14% ethanol; G: pH 3.2; H: pH 3.8.

Using the RTA values of growth curves, a data matrix was built and used to obtain a dendrogram based on Pearson's correlation coefficient and the unweighted pair group method with arithmetic average (UPGMA) as the agglomerative clustering (Vauterin and Vauterin, 1992) (Figure 2).

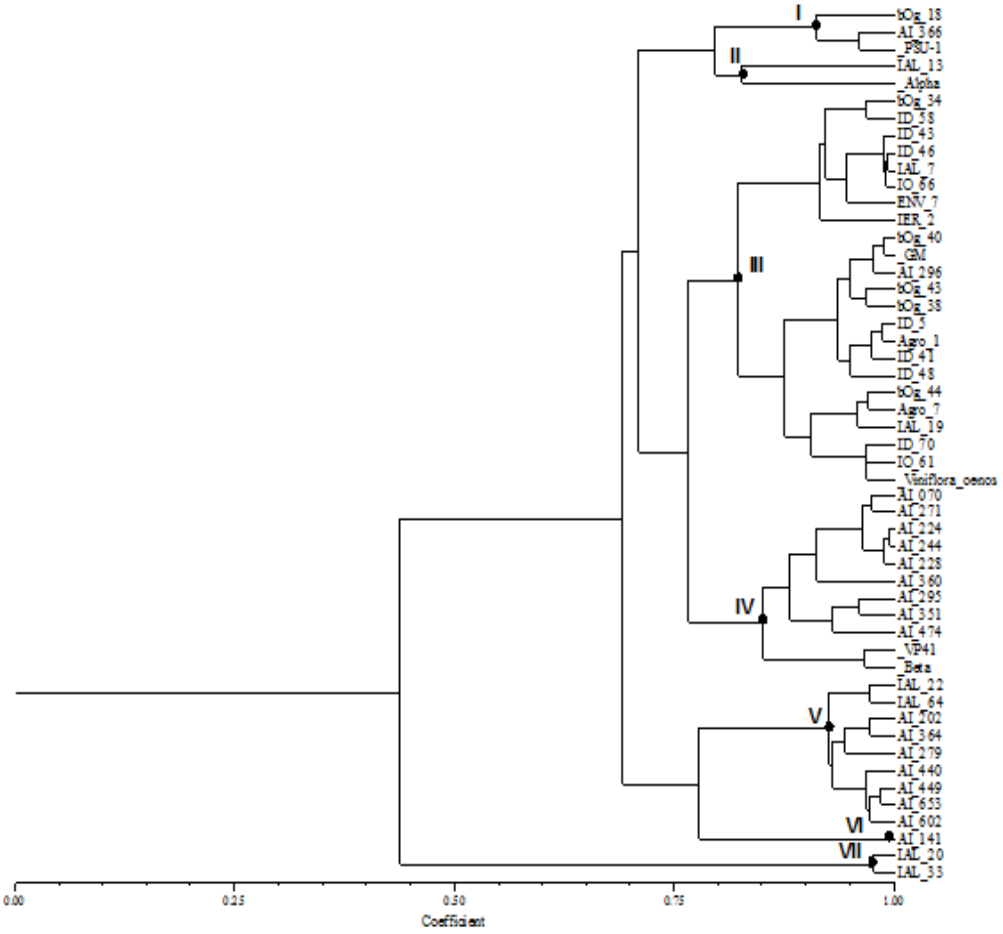

Figure 2 - Dendrogram constructed from the matrix of *O. oeni* strains versus trapezoidal areas under growth curve for the 12 tested assays, using the Pearson correlation coefficient and the agglomeration method UPGMA. The vertical dotted line indicates the similarity value (80%) used for the definition of each cluster.

Analysis of the dendrogram revealed that all strains clustered below 50% similarity, showing variability on growth behaviour under the selected conditions. At a similarity level of 80% seven clusters were defined, each including oenococci from different wine regions: (i) Cluster I grouped two *O. oeni* isolates from Dão and Douro region and PSU-1 starter; (ii) cluster II grouped one *O. oeni* isolate from Alentejo and Alpha starter; (iii) cluster III grouped one *O. oeni* from Douro, 11 from Dão, seven from Ribatejo, two from Alentejo and two commercial starters (GM and Viniflora oenos); (iv) cluster IV grouped nine *O. oeni* from Douro and two commercial starters (VP41 and Beta); (v) cluster V grouped seven *O. oeni* from Douro and two from Alentejo; (vi) cluster VI only included one *O. oeni* from Douro and (vii) cluster VII grouped two *O. oeni* isolates from Alentejo. These results showed that *O. oeni* strains from Dão and Ribatejo present the most similar growth behaviour and *O. oeni* strains from Douro and Alentejo present the most diverse growth behaviour.

To further explore the obtained results, a PCA analysis was also accomplished. The first three dimensions explained 73% of the total variance of the data. The first principal dimension, which explains 45% of the total variance, was correlated with culture medium A, E, F, G, H, I and J (see Table 3). The second principal dimension, which explains 18% of the total variance, was correlated with culture medium B, C and D (see Table 3). The third principal dimension, which explains 10% of the total variance, was correlated with culture medium K and L (see Table 3). In Figure 3 (A and B) strains are distributed along the axis according to their growth response gradient. Axis D1 is related to growth in “synthetic wine” at low pH and in the presence of ethanol. Major growth is observed in the positive extremity (strains GM and AI202) and the lowest growth in the negative extremity (strains AI366 and AI1653). Axis D2 is associated with growth in synthetic wine in the presence of SO_2. In the negative extremity of the axis are depicted strains with the highest growth (strains PSU-1 and VP41) and in the positive

extremity those with the lowest growth (strain AI141). Axis D3 is associated growth in culture medium MRS pH 5.5 supplied with 0.5 malic acid. Major strain growth is observed in the positive extremity (strains GM and PSU-1) and the lowest growth in the negative extremity (strains VP41 and ID58).

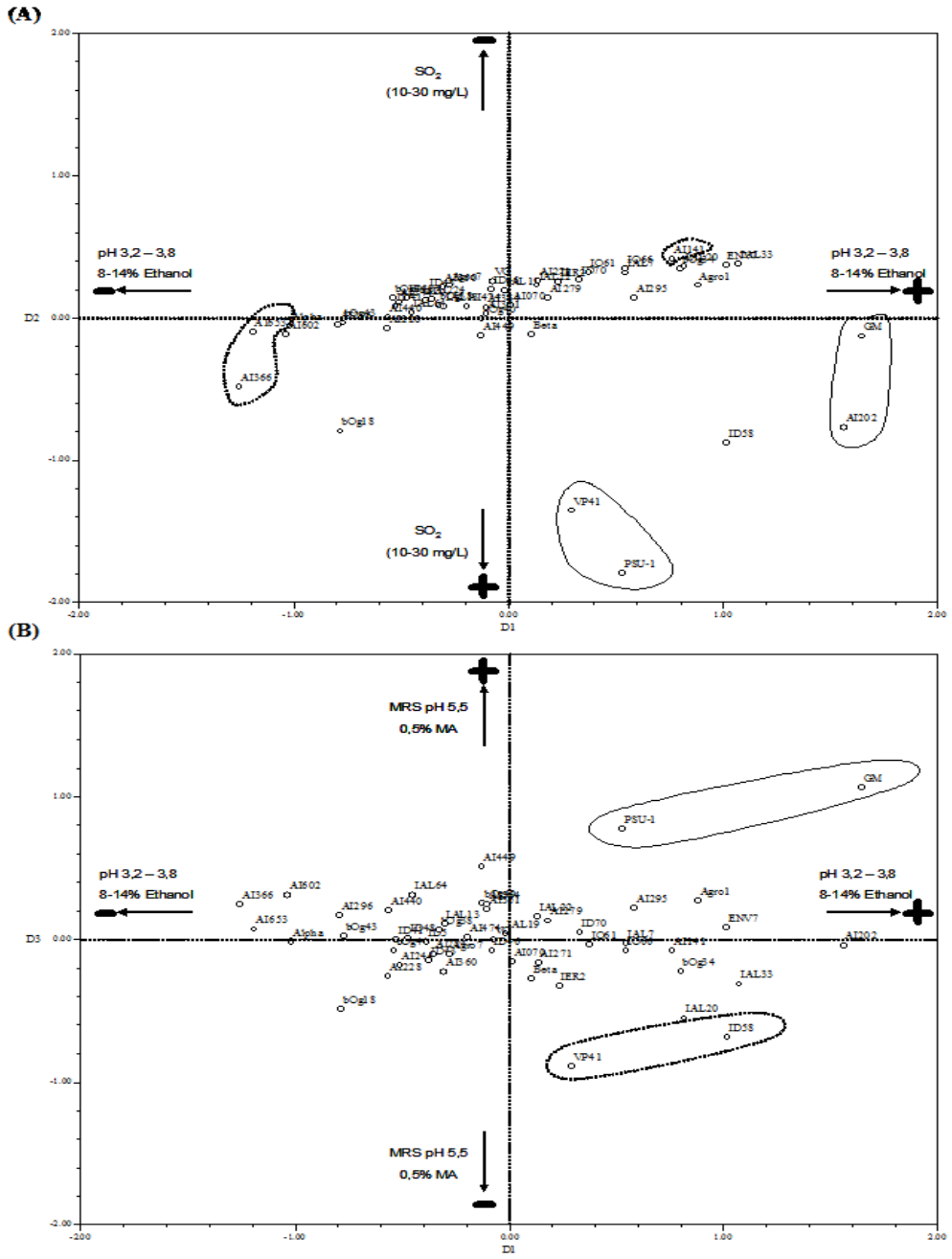

Figure 3 - Projection graphics obtained by PCA from the matrix of relative trapezoidal areas under growth curve for the 12 tested assays. A: Projection on axis D1 and D2; B: Projection on axis D1 and D3.

The analysis of the three dimensions plot showed that *O. oeni* strains of different winemaking regions and commercial malolactic starters can be grouped together.

Discussion

MLF, generally carried out by *O. oeni*, is a desired process for some wines, since it contributes for the deacidification of wine, ensures microbial stability and enhances the organoleptic properties of wine. The metabolic activity of some *O. oeni* strains may give rise to undesirable compounds, such as biogenic amines and ethyl carbamate precursors. Since consumers understandably look for safe quality products, in addition to high sensory quality, wine producers and winegrowers must pay increasing attention to such concerns. When the indigenous lactic microbiota of a cellar is known to comprise undesirable strains, their participation in malolactic fermentation can be prevented by use of malolactic starters. According to our results, the ability to decarboxylate amino acids and to degrade arginine should now be included in the selection criteria for new starters. However, it is also essential to select starters able to change positively the wine volatile fraction throughout the liberation of glycosidically bound aroma compounds and capable to tolerate the harsh physico-chemical conditions of wine.

Oenological characterization of 121 *O. oeni* isolates from wines of three different winemaking regions (Dão, Ribatejo and Alentejo) of Portugal was performed by the screening of malolactic and β-glucosidase activities, as well as production of biogenic amines and ethyl carbamate precursors. In order to assess functional behaviour, 51 *O. oeni* strains (26 *O. oeni* strains from Dão, Ribatejo and Alentejo wines, 19 *O. oeni* strains from Douro and six commercial malolactic starters) were tested for their capacity to grow at different conditions (pH, ethanol, SO_2, malic acid, temperature) in “synthetic

wine" and culture media, as an additional criterion to select the best *O. oeni* strains to be candidate as starters in vinification processes.

The ability to produce biogenic amines was evaluated using three different methods: plate medium, HPLC and PCR. Five LAB strains (two *L. brevis*, one *L. buchneri* and two *O. oeni*) were used as positive controls to check the feasibility of the methods. The decarboxylase medium described by Landete *et al*. (2005b) allowed the detection of ornithine decarboxylase activity since the amount of putrescine was high (>1000 mg/L). Cadaverine production was not detected, since the amount produced was low (below 20 mg/L), therefore not enough to induce a rise in the pH and a subsequent colour change in the media. These false negatives were revealed to be positives when using HPLC determination. This result is not surprising, as previous reports have shown that the plate medium assay, a qualitative method for detecting BA, is valid only when the level of BA in the medium exceeds 100 mg/L (Landete *et al*., 2005a). HPLC-mediated quantitative detection of BA in the supernatants of cultures grown in decarboxylase broth allowed the detection of putrescine and cadaverine-producing strains. However, putrescine and cadaverine production appears not to be a general trait among *O. oeni* isolates from Portuguese wines, as only up to 23% of the *O. oeni* isolates were putrescine producers and/or cadaverine producers. This work shows that the ability of *O. oeni* to produce putrescine and cadaverine is not an intrinsic characteristic of this species, rather behaving as strain-dependent. The inability of *O. oeni* strains to produce tyramine is in agreement with the results obtained by Moreno-Arribas *et al*. (2000, 2003) and Landete *et al*. (2007). However, some authors (Choudhury *et al*., 1990; Gardini *et al*., 2005; Garai *et al*., 2007) reported that *O. oeni* is able to decarboxylate tyrosine in a "synthetic medium". On another hand, several *O. oeni* strains were described as histidine decarboxylase positive (Funel and Joyeux, 1994; Coton *et al*., 1998; Lonvaud-Funel, 2001; Guerrini *et al*., 2002; Landete *et al*., 2005a). In contrast, formation of histamine was not observed in any species that may be involved

in malolactic fermentation (Straub *et al*., 1995; Moreno-Arribas *et al*., 2003). In this study, no *O. oeni* strain histamine producer was detected, thus suggesting that the presence of the histidine decarboxylase enzyme appears to be a uncommon episode in *O. oeni* isolates from Portuguese wines. These opposing results are probably due to the different microbial population present in grapes and wineries from different geographical regions and countries.

Decarboxylase activity seems thus to be strain dependent and it is conceivable that environmental conditions act as the main regulator factor at gene expression level, explaining the divergence of results reported by different authors.

Our results also show that the capacity to degrade arginine via ADI pathway appears to be widespread among strains of *O. oeni*, although low amounts of citrulline and carbamyl phosphate were produced from the degradation of arginine. Nevertheless, the capacity to degrade arginine should be carefully assessed and included among the selection criteria of malolactic strains to be used in winemaking.

Growth curves and subjacent mathematical models are useful to understand strain behaviour as conditions can be analysed independently from each other, giving information about ranges of growth and adaptability to each condition. Turbidimetric methods seem to be a good alternative to viable cell counts in order to study bacterial growth since OD measurement gives a real time measure of bacterial population and, despite the high threshold detection of turbidimetric devices, they have practical significance when dealing with bacteria in high cell densities (Dalgaard and Koutsoumanis, 2001). The multivariate statistical analysis allowed the selection of two *O. oeni* isolates from winemaking region of Dão and Douro (ID58 and AI202) as the most suitable regional *O. oeni* strains to be used as malolactic starters for the wine industry. Together with commercial malolactic starters VP41, PSU-1 and GM, these strains showed a high capability to grow in the presence of SO_2, high ethanol concentration and low pH.

In conclusion, several *O. oeni* strains isolated from Portuguese wines showed important properties for potential practical application as malolactic starters. In further investigations the behaviour of selected strains must be evaluated in wine cellar-scale in order to validate their applicability for winemaking.

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3.4 Supplementary data

Table 1 - Contents of biogenic amines in Dão wines used for *Oenococcus oeni* strains isolation.

Oenococcus oeni strain	Wine sample	Biogenic Amines (mg/l)*					
		Tyramine	Putrescine	Cadaverine	Histamine	Isoamylamine	Phenylethylamine
DS 5	DSNIFML	<0,040	2,260	<0,060	0,116		<0,030
ID 4	DVT1	2,016	6,193	<0,060	7,212	0,300	<0,030
ID 5	DVT1						
ID 6	D0905	<0,040	5,172	<0,060	0,292		<0,030
ID 38	DS1104	<0,040	1,348	<0,060	0,225		<0,030
ID 39	DS1007	0,862	6,402	2,021	0,270		< 0,030
ID 40	DS1806	<0,040	5,333	0,195	0,427		<0,030
ID 41	VTMEA1	<0,040	0,340	<0,060	0,119		<0,030
ID 42	DS903	<0,040	3,369	<0,060	0,194		<0,030
ID 43	DS1905	0,134	10,208	0,107	1,453	0,153	<0,030
ID 44	DS5007	0,306	3,677	<0,060	5,309	0,140	<0,030
ID 45	DS0805	<0,040	3,024	<0,060	1,058		<0,030
ID 46	DS1203	<0,040	2,443	<0,060	0,228		<0,030
ID 47	DS0208	0,328	5,420	0,150	0,240		<0,030
ID 48	DS1201	<0,040	3,037	<0,060	0,315		<0,030
ID 53	DS1201						
ID 55	DS1903	1,172	6,702	0,200	2,174	0,140	<0,030
ID 56	D2007	2,464	11,560	0,310	3,050		<0,030
ID 57	DS0801	<0,040	2,514	<0,060	0,204		<0,030
ID 58	DS1102	<0,040	0,805	<0,060	0,185		<0,030
ID 62	D0107	1,574	8,449	0,182	0,357		<0,030
ID 65	D0604	1,160	9,292	0,419	3,952	0,100	<0,030
ID 70	D2006	0,858	8,293	0,120	1,362		<0,030

Table 2 – Contents of biogenic amines in Ribatejo wines used for *Oenococcus oeni* strains isolation.

Oenococcus oeni strain	Wine sample	Biogenic Amines (mg/l)*					
		Tyramine	Putrescine	Cadaverine	Histamine	Isoamylamine	Phenylethylamine
EVN 1	VEVN1	<0,040	0,601	<0,060	<0,030	0,000	<0,030
EVN 2	VEVN2	<0,040	2,101	<0,060	<0,030	0,000	<0,030
ENV 7	VEVN1	1,539	14,341	0,345	16,210	0,720	0,147
E 169	VEVN9	<0,040	0,318	<0,060	<0,030	0,000	0,199
IO 1	VT1	4,565	<0,060	0,398	<0,030	6,880	<0,030
IO 2	VT2	0,428	0,075	0,213	<0,030	0,013	<0,030
IO 24	VT4						
IO 25	VT4	1,236	<0,060	0,117	<0,030	0,081	<0,030
IO 27	VT4						
IO 30	VT3	6,238	<0,060	0,629	<0,030	5,020	<0,030
IO 58	VB1	2,651	<0,060	0,475	0,186	0,763	<0,030
IO 59	VB9	2,594	<0,060	0,575	0,194	0,933	<0,030
IO 60	VB8	2,352	<0,060	0,813	0,176	0,881	<0,030
IO 61	VB10	2,704	<0,060	0,582	0,126	0,935	<0,030
IO 62	VB10						
IO 63	VB8	2,352	<0,060	0,813	0,176	0,881	<0,030
IO 64	VB3	2,508	<0,060	0,364	0,178	0,722	<0,030
IO 66	VB12	3,264	0,495	1,501	0,671	1,508	<0,030
IO 75	VB1	2,651	<0,060	0,475	0,186	0,763	<0,030
Agro 1	VEVNC01	6,400	0,095	0,402	0,152	1,651	2,950
Agro 2	VEVNCN01	10,651	0,098	1,015	0,186	4,816	3,367
Agro 3	VEVNCB01	6,455	0,084	0,373	0,134	1,615	2,866
Agro 4	VEVNCNB01	10,249	0,095	0,407	0,154	4,616	3,274
Agro 5	VEVNC02	1,898	<0,060	0,148	0,289	0,000	0,345
Agro 6	VEVNCN02	2,271	<0,060	0,162	0,922	0,069	0,383
Agro 7	VEVNCB02	2,544	<0,060	0,144	0,346	0,000	0,355
Agro 8	VEVNCNB02	3,214	<0,060	0,173	1,818	0,071	0,376
Agro 9	VEVNC04	2,434	<0,060	0,190	1,136	0,000	0,357
Agro 10	VEVNCN04	3,063	0,083	0,229	2,337	0,071	0,436
EVN 19	VEVN19	3,492	14,199	0,304	7,050	0,000	0,404
EVN 22	VEVN22	3,137	15,995	0,739	3,157	0,000	0,796
EVN 26	VEVN26	<0,040	3,831	0,202	0,723	0,000	<0,030
IER 1	Est/Rib2	<0,040	3,443	<0,060	<0,030	0,000	<0,030
IER 2	Est/Rib1	<0,040	4,662	0,293	<0,030	0,000	0,141
IER 3	Est/Rib4	<0,040	4,728	0,253	<0,030	0,000	<0,030

Table 3 - Contents of biogenic amines in Alentejo wines used for *Oenococcus oeni* strains isolation.

Oenococcus oeni strain	Wine sample	Biogenic Amines (mg/l)*					
		Tyramine	Putrescine	Cadaverine	Histamine	Isoamylamine	Phenylethylamine
IAL 7	VAL29	6,144	<0,060	0,819	9,617	0,000	0,141
IAL 8	VAL40	2,808	<0,060	0,544	4,899	0,000	<0,030
IAL 9	VAL20	3,021	<0,060	0,773	6,069	0,000	<0,030
IAL 10	VAL23	0,224	<0,060	<0,060	1,657	0,000	0,201
IAL 11	VAL16	1,193	<0,060	1,250	2,830	0,000	<0,030
IAL 12	VAL9	3,324	<0,060	1,214	6,880	0,000	<0,030
IAL 13	VAL8	0,277	<0,060	<0,060	<0,030	0,000	0,136
IAL 14	VAL16	1,193	<0,060	1,250	2,830	0,000	<0,030
IAL 15	VAL38	3,332	<0,060	<0,060	4,703	0,000	0,156
IAL 16	VAL33	4,806	<0,060	<0,060	7,498	0,000	<0,030
IAL 17	VAL31	3,670	<0,060	<0,060	6,615	0,000	<0,030
IAL 18	VAL27	4,914	<0,060	<0,060	9,255	0,000	0,161
IAL 19	VAL12	0,248	<0,060	<0,060	1,558	0,000	0,148
IAL 20	VAL21	0,225	<0,060	<0,060	1,516	0,000	0,147
IAL 21	VAL13	0,340	<0,060	<0,060	1,874	0,000	0,176
IAL 22	VAL18	5,281	<0,060	<0,060	<0,030	0,000	<0,030
IAL 23	VAL36	2,872	<0,060	0,575	4,584	0,000	<0,030
IAL 24	VAL26	4,322	<0,060	0,801	7,829	0,000	0,155
IAL 25	VAL35	3,613	<0,060	<0,060	6,688	0,000	<0,030
IAL 26	VAL14	0,226	<0,060	1,120	1,189	0,000	0,137
IAL 27	VAL17	3,375	<0,060	1,027	7,443	0,000	0,130
IAL59							
IAL 28	VAL30	5,051	<0,060	<0,060	8,155	0,000	<0,030
IAL 29	VAL28	5,403	<0,060	0,735	8,520	0,000	0,112
IAL 30	VAL5	< 0,040	8,962	0,562	<0,030	0,000	<0,030
IAL 31	VAL22	0,291	<0,060	<0,060	1,990	0,000	0,206
IAL60							
IAL 33	VAL4	0,126	<0,060	0,544	<0,030	0,000	<0,030
IAL 34	VAL19	4,295	<0,060	<0,060	7,771	0,000	<0,030
IAL 35	VAL7	0,307	<0,060	<0,060	<0,030	0,000	0,146
IAL 36	VAL37	3,184	<0,060	0,600	4,569	0,000	<0,030
IAL 37	VAL32	4,426	<0,060	<0,060	7,212	0,000	<0,030
IAL 49	VAL39	3,031	<0,060	0,507	5,040	0,000	<0,030
IAL 50	VAL10	4,318	<0,060	<0,060	9,635	0,000	<0,030
IAL 51	VAL2	0,256	<0,060	<0,060	<0,030	0,000	<0,030
IAL 52	VAL3	0,314	<0,060	0,687	0,439	0,000	<0,030
IAL 54	VAL24	0,150	6,628	<0,060	<0,030	0,000	<0,030
IAL 61	VAL24						
IAL 63	VAL5	< 0,040	8,962	0,562	<0,030	0,000	<0,030
IAL 64	VAL5	< 0,040	<0,060	<0,060	<0,030		<0,030
IAL66	VAL26	4,322	<0,060	0,801	7,829	0,000	0,155
IAL 71	VAL2	0,256	<0,060	<0,060	<0,030	0,000	<0,030

* Biogenic amines (tyramine, putrescine, cadaverine, histamine, isoamylamine and phenylethylamine) were analysed by reverse-phase high-pressure liquid chromatography (RP-HPLC) according to the method described by Vidal-Carou *et al*., (2003). The RP-HPLC analysis was carried out with a fluorescence detector (excitation wavelength of 340 nm, and emission wavelength of 425 nm). The separations were performed on a Waters Nova-Pack C18 column. The derivatization process was post-column performed with o-phtaldialdehyde/2-mercaptoethanol (OPA/MCE) reagent. Samples were filtered (0.45 µm pore size filter; Millipore, USA) and then directly injected in duplicate onto the HPLC system. All the reagents used were HPLC grade.

CHAPTER 4

TRANSCRIPTIONAL ANALYSIS OF *OENOCOCCUS OENI* STRAINS

The first part of the Chapter IV aims to analyze the transcriptome profile of four Portuguese autochthonous *Oenococcus oeni* strains and two malolactic starters by Random Arbitrarily Primed PCR under wines from different winemaking regions of Portugal in order to verify the applicability of the selected potential starters and to obtain a better stress response definition in this species. The second part of the Chapter IV focus on the study of the response of malolactic enzyme gene and arginine deiminase gene cluster in one *O. oeni* strain under the effect of different wine stresses by reverse transcription polymerase chain reaction, in order to evaluate the potential applicability of transcription studies in the process of malolactic starter selection.

This chapter consists of one scientific article:

Marques, A.P., San Romão, M.V., Tenreiro, R. RNA fingerprinting analysis of *Oenococcus oeni* strains under wine conditions (Submitted to *Food Microbiology*)

The experimental work presented in this chapter was done by the author. In the bacterial growth and RNA extraction and quantification the author had help of Diogo Pereira. The manuscript was written by the author and revised by the other co-authors of the article.

4.1 RNA fingerprinting analysis of *Oenococcus oeni* strains under wine conditions

RNA fingerprinting analysis of *Oenococcus oeni* strains under wine conditions

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ABSTRACT

Oenococcus oeni is a lactic acid bacterium of economic interest used in winemaking. This bacterium is the preferred species for malolactic fermentation (MLF) due its adaptability to the chemically harsh wine environment. MLF enhances the organoleptic properties and ensures deacidification and microbial stability of wines.

The aim of this work was the transcriptional characterization of six *O. oeni* strains, four of them selected from distinct winemaking regions of Portugal, as candidates to malolactic starters, and two commercial malolactic starters. Using crossed assays with wines from different Portuguese winemaking regions, strain characteristic transcriptional patterns induced by each wine were analyzed based on Random Arbitrarily Primed PCR (RAP-PCR).

The obtained results suggest that the starter strains showed more constrained and limited transcription profiles, whereas a high variation on the distribution of the transcription profiles was observed for the regional strains in each wine.

According with our results, RAP-PCR is a useful technique for a preliminary investigation of strain behavior under different wine environmental conditions, which can be applied in field studies to monitor differential patterns of global gene expression and to select markers for the surveillance of starters performance in winemaking, as well as for quality and safety control.

Keywords: Oenococcus oeni, wine, transcriptional analysis, random arbitrarily primed PCR.

1. Introduction

Malolactic fermentation (MLF) is one of the most difficult steps to control in winemaking. MLF, especially important for high acidic wines, decreases its total acidity, improves microbiological stability and enhances organoleptic properties. *Oenococcus oeni* is the lactic acid bacteria (LAB) mainly responsible by MLF. This species is normally well adapted to the harsh environmental conditions of wine (Lonvaud-Funel, 1999). In addition to LAB occurring naturally in wine, starter culture strains of *O. oeni* are often used during the winemaking process to improve the efficiency of the MLF. Differences between starter culture strains are related to their inherent stress resistance (Guzzo et al., 1998).

Molecular techniques known in general as RNA fingerprinting include differential-display PCR (DD-PCR) (Liang and Pardee, 1992; McClelland et al., 1995; Chia et al., 2001), fluorescent differential display (FDD) (Ripamonte et al., 2005; Sico et al., 2009; Bonomo et al., 2010) and random arbitrarily primed PCR (RAP-PCR) (Welsh et al., 1992; Wong et al., 1994; Shepard et al., 1999; Du and Kolenbrander, 2000; Frias-Lopez et al., 2004; Papadimitriou et al., 2008) and have become routine to examine changes in gene expression.

These molecular methods could be useful tools to identify differently expressed transcripts and to compare the difference of cDNA fingerprints when certain environmental conditions and time periods are involved, using small amounts of RNA (Colin and Olsen, 2001; Lockyer et al., 2004).

RAP-PCR utilizes an arbitrary primer at a low annealing temperature for both the first- and second-strand cDNA synthesis reactions (Welsh et al., 1992; Shepard and Gilmore, 1999). Differences in gene expression could be detected from the obtained fingerprinting pattern by observing the presence or absence of specific products obtained from different populations of cells (Shepard and Gilmore, 1999). In spite of its intrinsic variability, common to other random priming methods, this technique has been successfully applied to several prokaryotic systems (Wong and McClelland, 1994; Shepard and Gilmore, 1999; Papadimitriou et al., 2008; Garbeva and de Boer, 2009; Ferraz et al., 2010).

In the present study, the transcriptional profiles of *O. oeni* strains were characterized by RAP-PCR under environmental wine conditions, in order to analyze the effect of distinct wine matrices and to investigate the potential of this approach in the selection of starter strains.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Six *Oenococcus oeni* strains were selected, based on their enological characteristics (unpublished results), including four strains isolated from wines of different Portuguese winemaking regions, namely Douro (AI202), Dão (ID58), Ribatejo (Agro1) and Alentejo (IAL7) and two commercial malolactic starters (VP41 and PSU-1).

Strains were cultivated until the end of exponential phase in MRSm (MRS, Merck; pH 5.5 supplied with 0.5% malic acid) medium at 30ºC. The cells were harvested by centrifugation, washed with sterile distilled water and resuspended in sterile distilled water to approximately 10^8 cfu/mL. The bacterial suspension was used to inoculated the experimental media (wine from Douro, Dão, Ribatejo and Alentejo region and also MRSm) at a rate of 2% (v/v). Incubation was carried at 20ºC during two, four and eight days. The wines were previously sterilized by filtration through a 0.2 µm pore size membrane. The efficiency of the sterilization method was confirmed by plate inoculation (MRSm agar) followed by 8 days incubation at 30ºC.

2.2. DNA and RNA extraction, quantification and DNase treatment

Cells were harvested by centrifugation, pellets were washed twice with phosphate buffer saline (PBS) pH 7.0 at 10 mmol/L, resuspended in 250 μL Tris-EDTA with 10 mg/mL lysozyme and incubated at 37ºC for 1 h. DNA was extracted using an UltraClean™ Microbial DNA Isolation Kit (MO-BIO Laboratories, USA). RNA was extracted using Trizol® reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Briefly, cells were homogenized in 1 mL of Trizol® reagent and centrifuged at 12 000 g for 10 min at 4ºC. Samples were incubated at room temperature for 5 min followed by the addition of 200 μL chloroform, incubation at room temperature during 3 min and centrifugation at 12 000 g for 15 min at 4ºC. Aqueous phase was collected to a new diethylpyrocarbonate (DEPC) treated tube and the RNA was precipitated by the addition of 500 μL isopropyl alcohol followed by incubation at room temperature for 10 min and centrifugation at 12 000 g for 10 min at 4ºC. RNA was washed by gentle mixing with 1 mL 75% ethanol followed by centrifugation at 7400 g for 5 min at 4ºC. After air-drying, the purified RNA was ressuspended in 50 μL of DEPC-treated water. All RNA samples were quantified by measuring the absorbance at 260 nm with

Anthos Zenyth 3100 (Anthos Labtec Instruments, Salzburg, Austria). RNA samples of known concentration were used for calibration.

To avoid false positive amplification in reverse transcriptase PCR (RT-PCR), the residual contaminating DNA was removed by DNase treatment. For each sample, 3 μg of RNA were treated with 50 U DNase I (Invitrogen, Life Technologies) and incubated at 37ºC for 45 min. The reaction was stopped, after the addition of EDTA to a final concentration of 25 mmol/L, by heating at 65ºC for 15 min. The efficiency of the treatment was confirmed by negative PCR amplification of the housekeeping gene *rpo*B. After DNase treatment, RNA integrity was assessed by agarose gel electrophoresis; 1 μg of RNA of each DNase-treated sample were loaded on a 1% agarose gel in 0.5% TBE buffer at a voltage of 90 V for 2 h. The remaining RNA was stored at -80ºC and further used for cDNA synthesis.

2.3. RNA fingerprinting

RNA fingerprinting analysis was performed by random arbitrarily primed-PCR (RAP-PCR). A initial screening was performed with three RNA and three DNA samples using ten different primers: csM13 (Huey and Hall, 1989); 1281 and 1290 (Akopyanz et al., 1992); UBC275 (University of British Columbia, Canada); OPC19 (Operon Technology, USA); $(GACA)_4$ and $(GTG)_5$ (Meyer et

al., 1993); pA and pH (Ulrike et al., 1989); and JV17HC (Le Jeune et al., 1994). The selected primers for the RAP-PCR analysis were csM13, $(GTG)_5$ and pH.

All the primers, dNTPs and reagents used for RT-PCR were purchased from Invitrogen (UK) and reagents used for PCR amplifications were purchased from Bioron (Germany), with the exception of primers and dNTPs that were also from Invitrogen.

cDNA synthesis was performed using SuperScript™ III reverse transcriptase (RT) in accordance with the manufacturer's instructions, but with some modifications. Briefly, 210 ng of random primers, 0.1 mmol/L deoxynucleoside triphosphates (dNTPs) and sterile DEPC-treated water were added to 400 ng of total RNA to a final volume of 13 μL. The mixture was heated to 65ºC for 10 min, followed by incubation on ice during 1 min. Subsequently, 4 μl of 5× First-Strand buffer, 1 μL 0.1 mol/L dithiothreitol (DTT), 1 μL RNaseOUT™ recombinant RNase inhibitor (40 U/μL) and 0.5 μL of SuperScript™ III RT (200 U/μL) were added to a final volume of 20 μL. RT-PCR reactions were performed in a T Personal Themocycler (Biometra, Germany) as follows: incubation at 25°C for 5 min and at 50ºC for 45 min. Upon completion of first-strand cDNA synthesis, the reaction was heated to 70°C for 15 min to inactivate RT and then immediately stored at -20ºC.

DNA and cDNA were amplified using a reaction mixture containing 2.5 μL of 1× PCR buffer, 0.65 μl of $MgCl_2$, 0.5 μL of dNTPs (10 mmol/L), 1 μl of one random primer (csM13 or $(GTG)_5$ or pH) (50 μM), 2.5 μl BSA (10×), 1.25 μl DMSO, 0.2 μL of DFS Taq DNA polymerase (5 U/μL) and 1 μL of cDNA or DNA in a final volume of 25 μL. The reaction was incubated at 95°C for 5 min and the following parameters were used for 40 cycles of PCR: 94°C 1 min, 40°C 1 min, 72°C 2 min; a final extension was performed at 72°C for 10 min. Upon completion, the reaction was stored at 4°C. The RAP-PCR products were firstly visualized on 1% agarose gel (to confirm the presence of amplification products) and then resolved on a 6% polyacrylamide gel prepared in TBE buffer. Electrophoresis was performed at 60 V during 3 h and the agarose and polyacrylamide gels were stained with ethidium bromide and silver nitrate, respectively.

2.4. Data analysis

A database of the RAP-PCR patterns was created with Bionumerics software (version 3.0, Applied Maths, Ghent, Belgium). The gel photos were scanned and imported into a Bionumerics database as inverted 8-bit grey-scale TIF images. This software was used to normalize and analyze the RAP-PCR profiles obtained with each one of the three selected random primers. Each

band in RAP-PCR profiles was marked in order to create a binary data matrix based on presence (1) absence (0) of this transcript.

A global data matrix was obtained by concatenation of the data matrices of each primer (csM13, $(GTG)_5$ and pH) and multivariate statistical analyses were applied including both a hierarchical custering (HC) and a Factorial Correspondence Analysis (FCA), performed with NTSYSpc software (version 2.20d; Exeter Software). Regarding HC, dendrograms for strain, wine/culture medium and time-point, as well as a global one, were computed using the Dice similarity coefficient as association measure and the unweighted pair group method with arithmetic average (UPGMA) as the agglomerative clustering (Vauterin and Vauterin, 1992). For FCA, two factor plots were constructed and scatter projection diagrams were obtained both for strains and wine/culture medium.

To assess the diversity of RAP-PCR profiles, the indexes of Simpson (Hunter and Gaston 1988) and Shannon (Zar, 1984) were used. These indexes, that measure the probability of two profiles being different and express the observed diversity relatively to the maximum, respectively, were applied to the dendrograms constructed for each strain, wine/culture medium and time-point.

3. Results and Discussion

RAP-PCR is a powerful method that can be applied to identify differentially expressed genes. Differences in gene expression can be detected using this methodology, as each sample generates its own unique RNA fingerprint for each condition examined. The aim of the present work was the transcriptional characterization of four autochthonous *O. oeni* strains and two commercial malolactic starters under wine environmental conditions using this approach.

Different authors have used RAP-PCR to analyze differences in gene expression in prokaryotes. However, all of those experiments used cultures grown in laboratory media (Shepard and Gilmore, 1999; Chakrabortty et al., 2000; Du and Kolenbrander, 2000; Bidle and Bartlett, 2001; Habe et al., 2008; Papadimitriou et al., 2008; Garbeva and de Boer, 2009). To our knowledge, this is the first report that accomplished a transcriptional characterization of different *O. oeni* strains (isolates from wines of different winemaking regions and malolactic starters) after growth in wines from different winemaking regions.

The first cDNA strand was obtained by using random hexamers. In all cases, these short random primers provided cDNA for a large number of different genes. In combination with the cDNA products, a total of 10 different random primers were used. The tested primers can be classified into four groups:

primers directed to regions flanked by or containing micro-/mini-satellites [csM13; $(GTG)_5$; $(GACA)_4$]; random primers (OPC19, 1281, 1290, UBC275); universal primers for 16S rRNA (pA, pH); and primers for specific genes (JV17HC). Since preliminary tests showed that primers csM13, $(GTG)_5$ and pH provided a larger number of amplicons, as well as more consistent patterns, these three primers were selected for RAP-PCR experiments.

Nonradioactive methods have previously been used successfully to analyze differences in gene expression (Chen and Peck, 1996; Rompf and Kahl, 1997; Bockelmann et al., 1999; Ahmed et al., 2000). Both 1% agarose and 6% polyacrylamide gels were used with good results to analyze RNA fingerprints for our samples. Nevertheless, polyacrylamide gels were more sensitive, showing a larger number of different bands, as well as a higher resolution and a better definition of the transcriptional profiles. Therefore, this methodology was selected for RNA fingerprinting analysis.

Since six strains (four regional isolates, AI202 from Douro, ID58 from Dão, Agro1 from Ribatejo and IAL7 from Alentejo; and two commercial starters, VP41 and PSU-1) were inoculated in five distinct conditions (Douro, Dão, Ribatejo and Alentejo wines, as well as MRSm medium) and three time-points were analyzed (2, 4 and 8 days of incubation), a total of 90 RAP-PCR RNA patterns were obtained with each primer.

The analysis of the global dendrogram obtained for these 90 (6 strains x 5 wine/medium x 3 time-points) transcription profiles (resulting from the concatenation of the csM13, $(GTG)_5$ and pH RAP-PCR patterns) did not reveal any association between strains or wines (data not shown), due to the complexity and high diversity of profiles. Thus, partial dendrograms were built by strain, by wine/medium and by incubation period.

Figure 1 illustrates the diversity of RAP-PCR RNA fingerprints at strain (A), wine (B) and incubation period (C) levels. Figure 1A represents the RNA profiles of isolate IAL7, obtained from a wine of Alentejo region, cultured in all the wines under study, and in the MRS medium during 2, 4 and 8 days. Figure 1B represents the RNA profiles of the six strains incubated (AI202, ID58, Agro 1, IAL7, VP41 and PSU-1) in Douro wine for the same time-periods. Figure 1C represents the RNA profiles of the six strains in distinct wines and MRSm medium after 8 days of incubation. In the gel some common bands appeared in all of the samples independent to the strain and wine/culture medium (*v.g.* Figure 1A primer pH; Figure 1B with the three primers; Figure 1C especially evident with primers pH and $(GTG)_{5.}$). From the analysis of Figure 1A was possible to observe that transcriptional profiles from strain IAL7 (isolated from a Alentejo wine) were more similar when grown in Alentejo wine and more distinct when grown in Ribatejo wine. The analysis of Figure 1B shows that transcriptional profiles from Douro wine were more

similar for starter VP41 and more distinct for strain AI202 (isolated from Douro wine). Figure 1C allowed to conclude that, for the incubation period of eight days, the transcriptional profiles were more similar in Ribatejo wine and more distinct in Douro wine and also that the transcriptional profiles were more similar in starter VP41 and more distinct in strain AI202 (isolated from Douro wine) and starter PSU-1. Although the differences in the band profiles could be attributed to the distinct wine environment conditions, some common bands appeared in all samples independently of the strain and wine/culture medium. These bands may correspond to housekeeping genes or genes associated with metabolic pathways common to growth/adaptation in wine.

The high diversity of RAP-PCR profiles was further highlighted by the analysis of Simpson's (D) and Shanon-Wiener (J') indexes of diversity, using a cut-off value of 40% similarity for each dendrogram (by strain, by wine/culture medium and by incubation period). In fact, D values range from 0.81 to 0.96 and J' values from 0.65 to 0.98. Although the ranges overlapped, the highest variation was observed for strains ($0.81<D<0.96$; $0.65<J'<0.98$), followed by wine/culture medium ($0.90<D<0.94$; $0.86<J'<0.95$) and incubation period ($0.91<D<0.93$; $0.89<J'<0.95$).

To further explore the obtained results, a FCA was performed and the dispersal areas of the RAP-PCR RNA fingerprints obtained for each autochthonous strain (AI202, ID58, Agro1 and IAL7) and for the starters VP41 and PSU-1

were compared (Figure 2). Similarly, the dispersal areas of RAP-PCR RNA fingerprints for each wine (Douro, Dão, Ribatejo and Alentejo) and also for the culture medium were also analyzed (Figure 3).

From the analysis of Figure 2 was possible to observe that the transcription profiles of *O. oeni* regional strains seem to be affected by the different wines, presenting high variation on the distribution of the transcription profiles. Contrarily, *O. oeni* starter strains (VP41 and PSU-1) were not as affected as the regional strains by the wine environment, showing more constrained and limited transcription profiles for each wine. The regional Agro1 strain (isolated from Ribatejo) presents the most heterogeneous transcription profiles in the tested wines. The analysis of Figure 3 shows that all *O. oeni* strains demonstrated higher variable transcriptional profiles when growth in any of the wines studied than in the culture medium. Furthermore, there is a wider variability of the transcriptional profiles in Ribatejo wine than in the other ones, especially if comparing with those in Dão wine.

The obtained results point to a clear influence of the matrix in which the bacteria are cultured. In fact, the influence of several factors like pH, ethanol, malic acid content and also the polyphenolic wine composition is well documented as it was largely studied by the scientific community. This fact is particularly illustrated by the results obtained from the RAP-PCR transcription profiles in Ribatejo wines, which are generally more acidic and have a

significant content in polyphenolic compounds, especially if compared to Alentejo ones (lower acidity, higher ethanol content, and having a more balanced color composition). These two wines are exactly those showing the more distinct results concerning the transcription profiles, namely for the strains isolated from each one of these wines (Agro1 and IAL7 respectively). This seems to allow the conclusion that regional strains will be more adequate for fermenting the correspondent wines. Moreover, it appears that wines considered as having a more balanced composition, sometimes associated to higher quality, do not need a large expression of especial strain properties. Also a better adaptation of Alentejo isolated strains to their natural habitat may be admitted. This assumption seems to be corroborated by the behavior of the commercial starters, namely PSU-1, a strain with a large conservation time under laboratory conditions. However this observation does not allow any conclusion concerning either the bacteria performance or final product quality.

The results reported above clearly show that transcriptional analysis can be used as a powerful tool to evaluate the bacteria cell (namely *O. oeni*) adaptation to a set of environmental stress factors, thus appearing as an additional tool to be explored in *O. oeni* selection, in the case to perform wine malolactic fermentation.

In addition, FCA proved to be a useful statistical tool for comparing the similarity of the transcriptional profiles of each strain obtained for the different environmental wine conditions.

The normalized values of RAP-PCR products detected along incubation time (2, 4 and 8 days) are depicted in graphics of Figure 4. The number of amplicons showed in each graphic was normalized relatively to the number of amplicons detected in the culture medium for the same strain in the same wine at the same incubation time. Obviously, the pattern in the culture medium is always the same and equal to 1. VP41 and PSU-1 appear as having the small number of amplicons, that in some wines and/or incubation time are even fewer than in the MRSm medium.

In what concerns to the Douro strain AI202, it was possible to observe that the transcription quantitative pattern (number of transcripts) was similar in all wines, with exception for Douro wine, in which a decrease of transcripts along incubation time, was detected. In general, when compared with the other strains, ID58 (Dão) presented the highest number of transcripts for days four and eight, especially considering the Ribatejo and Alentejo wines. The generated data showed that, in general, the quantitative patterns of Agro1 strain from Ribatejo were lower than the patterns observed for the other strains.

The distribution of the transcription quantitative patterns (number of transcripts) observed for each strain in the same wine at the three different

incubation times was more homogeneous than the distribution of the transcription quantitative patterns across the different wines. These data suggest that, apparently, the number of transcripts detected along the incubation time is strain dependent and not wine related.

The problem concerning the use of universal starters versus regional starters to induce the malolactic fermentation in winemaking is not completely understood, and remains as a major subject for the improvement of wine quality and safety. Correlating multiple differences in the transcriptome of several *O. oeni* strains in different wines and medium conditions enables the achievement of robust information that may be used for selection of the most suitable strains to induce the malolactic fermentation.

The present study developed a rapid and accurate methodology by which the transcription profiles of *O. oeni* were analyzed. The generated data showed that the analysis of the transcriptome by RAP-PCR suggests that the transcription profiles of universal starters (PSU-1 and VP41) are not uniform when inoculated in wines from different winemaking regions of Portugal, therefore pointing to a distinct behavior when compared to the one of the regional strains. Although the wine matrix appears to be the dominant factor in gene expression, the behavior of each strain seems to be dependent on its gene pool. Thus, this behavior may be associated with differential gene expression pools induced by differences in the wine matrix. Alternatively, the use of a

diversified group of starters may provide a promising approach to induce the malolactic fermentation.

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Figure legends

Fig. 1. Dendrograms illustrating the diversity of RAP-PCR RNA fingerprints at strain (A), wine (B) and incubation period (C) levels. Similarity was calculated with Dice coefficient and agglomeration was based on UPGMA. Each fingerprint results from the concatenation of csM13, $(GTG)_5$ and pH RAP-PCR patterns. (A) RNA profiles of isolate IAL7, from Alentejo region, in distinct wines and time-periods. (B) RNA profiles of the six strains incubated in Douro wine for different time-periods. (C) RNA profiles of the six strains in distinct wines and MRSm medium at 8 days of incubation.

A three-digit code was used for each experimental variant. The first digit identifies the strain (1: AI202, Douro; 2: ID58, Dão; 3: Agro1, Ribatejo; 4: IAL7, Alentejo; 5: VP41 starter; 6: PSU-1 starter), the second identifies the wine/medium (1: Douro wine; 2: Dão wine; 3: Ribatejo wine; 4: Alentejo wine; 5: MRSm medium) and the third identifies the incubation time (1: 2 days; 2: 4 days; 3: 8 days).

Fig. 2. Two-factor plots depicting the dispersal area of RAP-PCR RNA fingerprints of each strain. The 90 experimental variants (6 strains x 5 wines/medium x 3 time periods) are projected in the Factor 1 x Factor 2 and Factor 1 x Factor 3 planes resulting from a Factorial Correspondence Analysis performed on the boolean matrix of the concatenated

csM13, $(GTG)_5$ and pH RAP-PCR patterns. The vertices of each polygon correspond to the RNA fingerprints observed for a particular strain in the four wines at 8 days of incubation. Small circles correspond to the RNA fingerprints of each strain in the culture medium MRSm at 8 days of incubation.

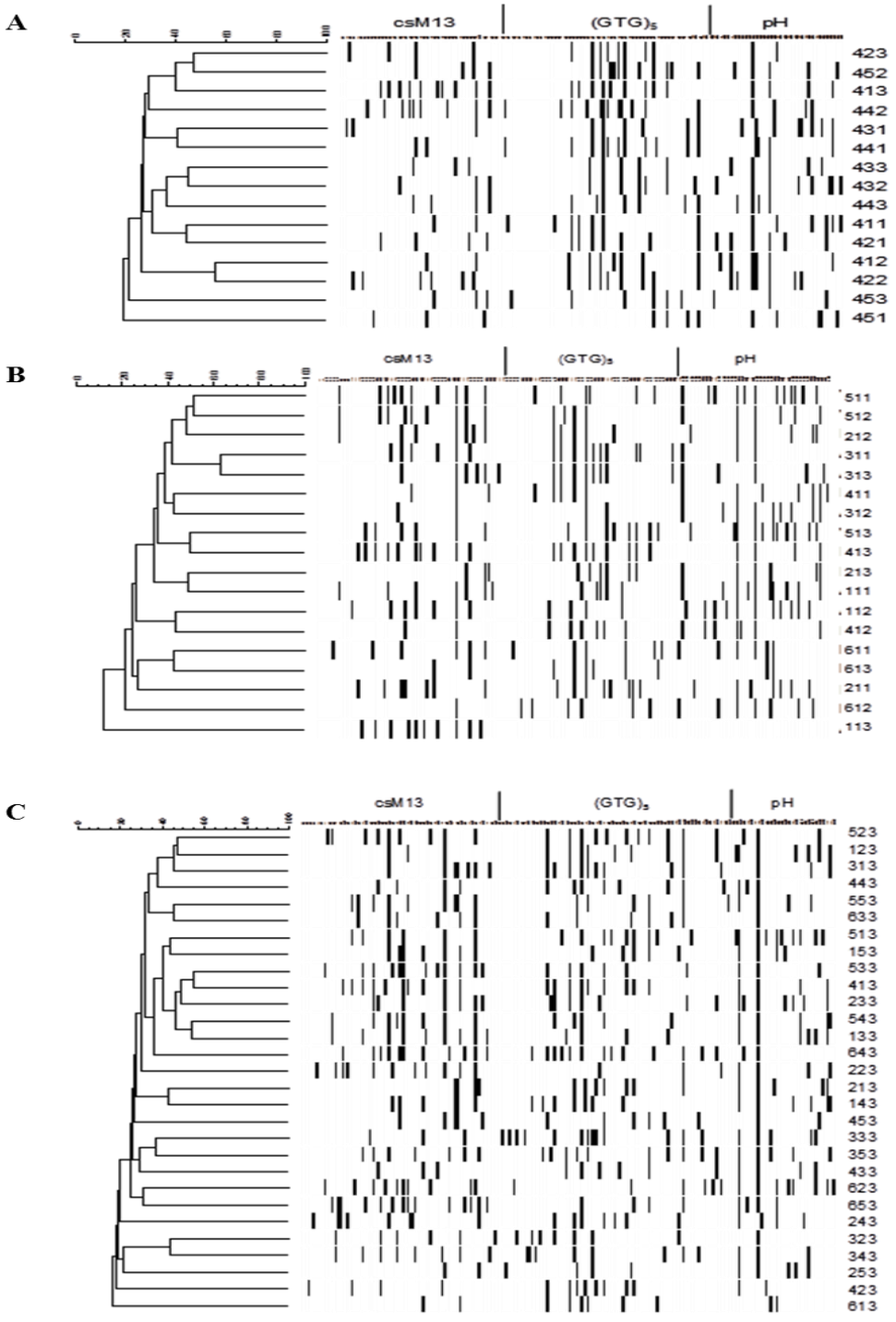
A three-digit code was used for each experimental variant. The first digit identifies the strain (1: AI202, Douro; 2: ID58, Dão; 3: Agro1, Ribatejo; 4: IAL7, Alentejo; 5: VP41 starter; 6: PSU-1 starter), the second identifies the wine/medium (1: Douro wine; 2: Dão wine; 3: Ribatejo wine; 4: Alentejo wine; 5: MRSm medium) and the third identifies the incubation time (1: 2 days; 2: 4 days; 3: 8 days).

Fig. 3. Two-factor plots depicting the dispersal area of RAP-PCR RNA fingerprints for each wine and the culture medium. The 90 experimental variants (6 strains x 5 wines/medium x 3 time periods) are projected in the Factor 1 x Factor 2 and Factor 1 x Factor 3 planes resulting from a Factorial Correspondence Analysis performed on the boolean matrix of the concatenated csM13, $(GTG)_5$ and pH RAP-PCR patterns. The vertices of each polygon correspond to the RNA fingerprints observed for each regional strain in a particular wine (or in the culture medium) at 8 days of incubation. Small circles linked by a colour line correspond to the RNA fingerprints of both starter strains in a particular wine (or in the culture medium) at 8 days of incubation.

A three-digit code was used for each experimental variant. The first digit identifies the strain (1: AI202, Douro; 2: ID58, Dão; 3: Agro1, Ribatejo; 4: IAL7, Alentejo; 5: VP41 starter; 6: PSU-1 starter), the second identifies the wine/medium (1: Douro wine; 2: Dão wine; 3: Ribatejo wine; 4: Alentejo wine; 5: MRSm medium) and the third identifies the incubation time (1: 2 days; 2: 4 days; 3: 8 days).

Fig. 4. Evolution of the number of amplicons in the RAP-PCR RNA fingerprints along incubation time (2, 4 and 8 days). For each strain in each wine/culture medium at each incubation time, the values were normalized relatively to the number of amplicons observed in the culture medium for the same strain in the same wine at the same incubation time.

Figure 1


A
csM13
(GTG)5
pH
423
452
413
442
431
441
433
432
443
411
421
412
422
453
451
B
csM13
(GTG)5
pH
511
512
212
311
313
411
312
513
413
213
111
112
412
611
613
211
612
113
C
csM13
(GTG)5
pH
523
123
313
443
553
633
513
153
533
413
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623
653
243
323
343
253
423
613

Figure 2

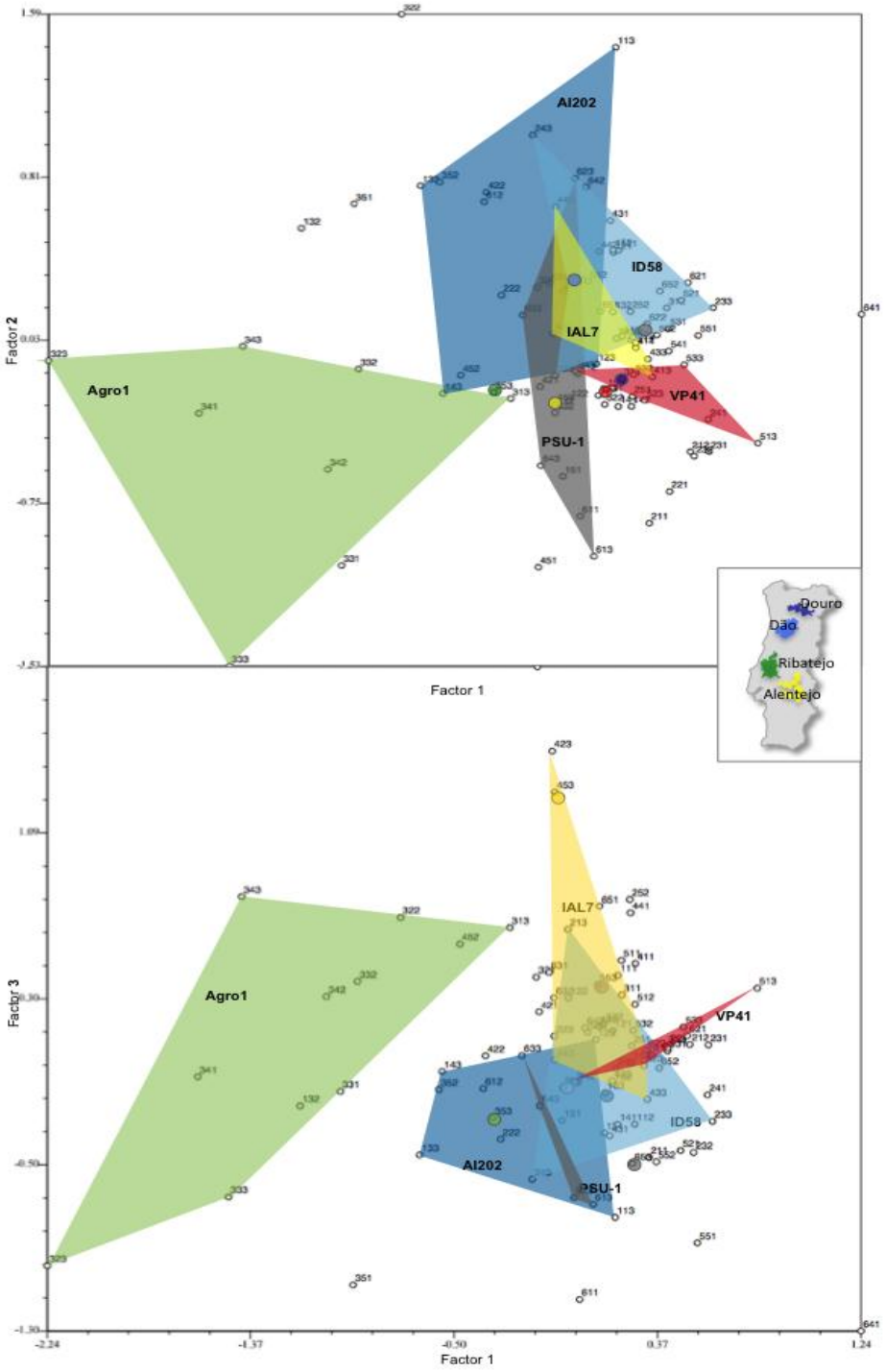

AI202
ID58
IAL7
VP41
PSU-1
Agro1
Douro
Dão
Ribatejo
Alentejo
Factor 1
Factor 2
Factor 3
1.59
0.81
0.03
-0.75
-1.53
1.09
0.30
-0.50
-1.30
-2.24
-1.37
-0.50
0.37
1.24

Figure 3

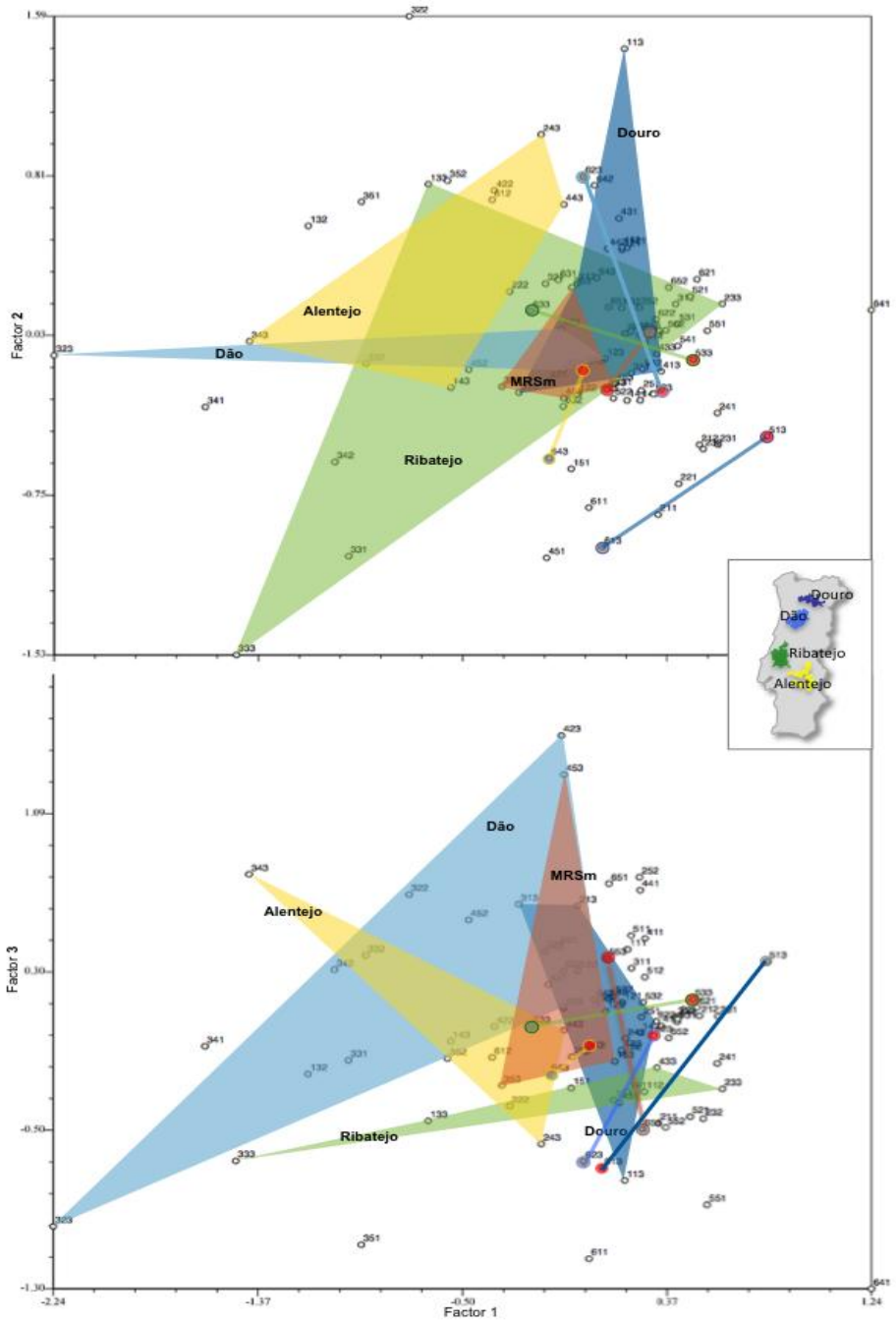
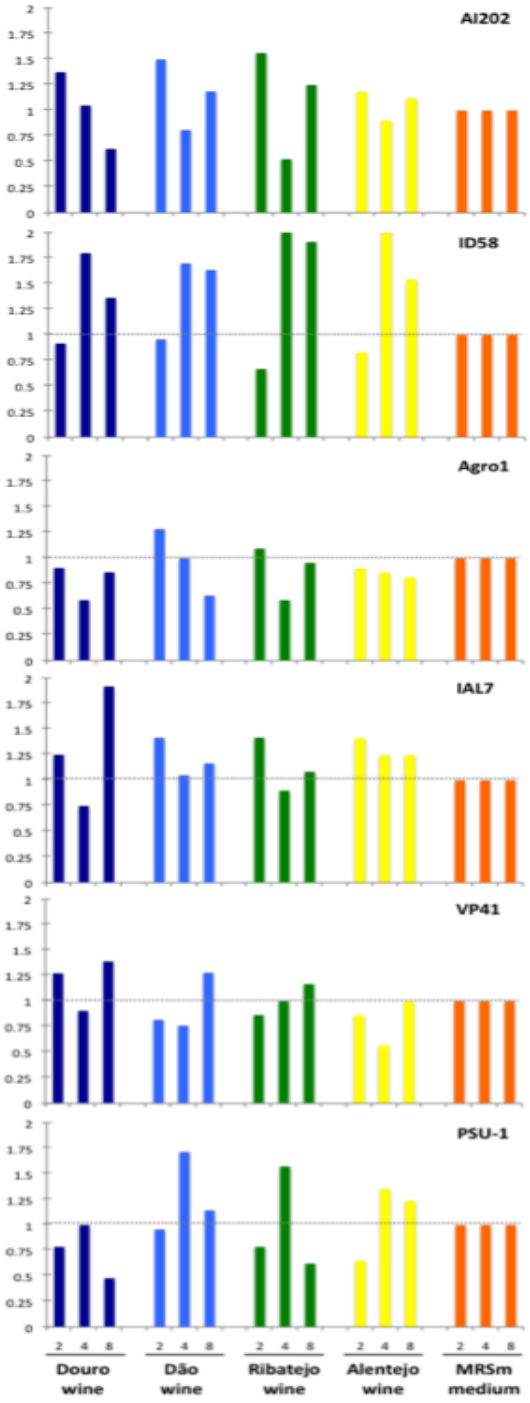

Douro
Alentejo
Dão
MRSm
Ribatejo
Factor 2
Factor 3
Factor 1
Douro
Dão
Ribatejo
Alentejo

Figure 4


AI202
ID58
Agro1
IAL7
VP41
PSU-1
2 4 8
Douro wine
Dão wine
Ribatejo wine
Alentejo wine
MRSm medium

4.2 Influence of malic acid and pH, ethanol and sulphur dioxide stresses on the expression of the malolactic enzyme gene and arginine deiminase gene cluster in *Oenococcus oeni*

Influence of malic acid and pH, ethanol and sulphur dioxide stresses on the expression of the malolactic enzyme gene and arginine deiminase gene cluster in *Oenococcus oeni*

Summary

Malolactic enzyme is the key enzyme which catalyses the convertion of L-malate into L-lactate during wine malolactic fermentation conducted by *Oenococcus oeni*. Arginine is a major amino acid in wine and its metabolism is particularly important in bacteria because it is an energy-yielding process. *O. oeni* is able to break down arginine through the arginine deiminase pathway. However, this mechanism can lead to the production of ethyl carbamate and putrescine, compounds that are known as having a negative impact on human health. The aim of this work was to study the effect of malic acid, pH, ethanol and SO_2 on malolactic enzyme gene (*mle*A) and arginine deiminase gene cluster (*arc*AC) in *O. oeni* using a transcriptional analysis. Both *mle*A and *arc*AC showed down-regulation for low concentrations of malic acid and low pH, as well as up-regulation for high ethanol content. High levels of SO_2 reduced *mle*A and increased *arc*AC expression. The observed results are here considered as adaptive responses to compensate possible inhibitory effects of the environmental conditions on the physiological activities of the bacterial cells. Intermediate down-regulation of *mle*A and no expression of *arc*AC was found in synthetic wine, thus pointing to the existence of fine-tuning mechanisms of gene expression as well as to the relevance of transcriptional studies as criteria in the selection of malolactic starters.

Introduction

The lactic acid bacterium *Oenococcus oeni* is widely used as starter to induce the malolactic fermentation (MLF) in wine industry, because it can tolerate the harsh physico-chemical conditions of wine. Malolactic enzyme is the key enzyme which catalyses the conversion for L-malate into L-lactate during MLF, that enhances the organoleptic properties and ensures deacidification and microbial stability of wines (Izquierdo Cañas *et al*., 2008; Lonvaud-Funel, 1999). The genes involved in the malolactic reaction are *mle*A that encodes the malolactic enzyme, *mle*P that encodes the malate permease and *mle*R (malolactic regulator) that encodes a regulatory protein (Bartowsky, 2005).

Arginine is one of the major components of the free amino nitrogen found in grapes and wine (Ribéreau-Gayon *et al*., 2006). Some amino acids, like arginine, may play a role in pH homeostasis (Tonon and Lonvaud-Funel, 2000). Furthermore, amino acid metabolism by lactic acid bacteria under the wine harsh conditions represents an important additional energy supply (Saguir and Manca de Nadra, 2002).

O. oeni metabolizes arginine via the arginine dihydrolase or deiminase (ADI) pathway (Liu and Pilone, 1998). ADI pathway is widely spread among prokaryotic organism (Cunin *et al*., 1986; Zúñiga *et al*., 1988) and comprises three enzymes: ADI (EC 3.5.3.6), which degrades arginine into citrulline and ammonia; ornithine transcarbamylase (OTC) (EC 2.1.3.3), that cleaves citrulline into carbamyl phosphate and ornithine; and carbamate kinase (CK) (EC 2.7.2.2), which produces ATP, ammonia, and carbon dioxide through dephosphorylation of carbamyl phosphate (Mira Orduña *et al*., 2000). This pathway is particularly important under environmental stress conditions after wine malolactic fermentation accomplishment, providing additional energy supply for bacterial growth (Konings *et al*., 1989; Marquis *et al*., 1987; Poolman *et al*., 1987; Thomas and Batt, 1968; Tonon and Lonvaud-Funel, 2000). However, this process is not necessarily desirable, since can lead to

the production of ethyl carbamate precursors (citrulline and carbamyl phosphate) and putrescine precursor (ornithine) (Arena and Manca de Nadra, 2005; Liu *et al*., 1995; Liu *et al*., 1996; Lonvaud-Funel, 1999), those compounds being considered as having undesirable physiological effects in human health, either by themselves or due to additive effects to other compounds (*e.g.* histamine) (Landete *et al*., 2007). The ADI cluster in *O. oeni* has been object of several studies and is commonly accepted that it comprises *arc*A (ADI), *arc*B (OTC), and *arc*C (CK), and two duplicated *arc*D genes (putatively coding for membrane proteins involved in arginine transport) (Arena *et al*., 1999; Divol *et al*., 2003; Liu and Pilone, 1998; Mangani *et al*., 2005; Tonon *et al*., 2001a, b).

The aim of this work was to study the response of malolactic enzyme gene and arginine deiminase gene cluster in an *O. oeni* strain (isolated from a Portuguese wine) under the effect of different wine stresses (malic acid, pH, ethanol and sulphur dioxide) by reverse transcription polymerase chain reaction (RT-PCR), in order to evaluate the potential applicability of transcription studies in the process of malolactic starter selection.

Material and methods

Bacterial strain and growth assays

O. oeni strain ID57, previously isolated by Marques *et al*. (2011) from a Portuguese wine of Dão region, was used in this study.

Ten different growth assays were performed using MRS broth (Merck) supplied with 5 g/L of arginine (assays A to I) and a 'synthetic wine' with 10% ethanol at pH 3.2 (assay J). The 'synthetic wine' was prepared as described by Liu *et al*. (1995) and sterilized by filtration through a 0.2 µm pore size membrane. The assayed conditions in MRS medium are fully described in Table 1.

Ethanol, malic acid and potassium metabisulphite ($K_2S_2O_5$) solutions were also sterilized by 0.2 µm membrane filtration. The efficiency of the sterilization methods was confirmed by plate inoculation (MRS agar, Merck) followed by incubation for 8 days at 30°C.

Cells were first pre-cultured in MRS broth pH 5.5, at 30°C until maximal growth. The cells were harvested by centrifugation, washed and resuspended in sterile distilled water. The cellular suspension was used as inoculum for all assays at an optical density of 0.1. The inoculated media were incubated at 30°C until the mid-stationary growth phase, assessed by absorbance follow-up at 600 nm. After growth, cells were collected by centrifugation at 4000*g* during 10 min and used for gene expression analysis.

Table 1 - Growth assays using MRS broth supplied with 5 g/l of arginine. For each factor the performed comparison is underlined.

Factor	Assay								
	A	B	C	D	E	F	G	H	I
pH	3.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Malic acid (%)	0.5	0.5	0.1	0	0.5	0.5	0.5	0.5	0.5
Ethanol (%)	0	0	0	0	11	14	0	0	0
SO_2 (mg/L)	0	0	0	0	0	0	10	20	30

DNA and RNA extraction, quantification and DNase treatment

Genomic DNA was extracted from cultures grown in MRS broth medium pH 5.5 until stationary phase at 30ºC. Cells were recovered by centrifugation and total DNA was obtained using an UltraClean™ Microbial DNA Isolation Kit (MO-BIO Laboratories, USA). Total RNA was extracted using Trizol® reagent (Invitrogen, USA) according to the manufacturer's instructions.

DNA and RNA samples were quantified by measuring the absorbance at 260 nm with Anthos Zenyth 3100 (Anthos Labtec Instruments, Salzburg, Austria).

To avoid false positive amplification in RT-PCR, the residual contaminating DNA was removed by DNase treatment using DNaseI (Invitrogen, USA) according to the manufacturer's recommendations. After DNase treatment, RNA integrity was assessed by 1% agarose gel electrophoresis (90 V; 1 h) and the remaining RNA was stored at - 80ºC and further used for cDNA synthesis.

RT-PCR analysis

cDNA synthesis was performed as follows: 400 ng of total RNA, diluted in DEPC-treated water, 1 μl of 1.25mM of each deoxynucleoside triphosphate (dNTP) and 1 μl of 300 ng/μl of random primer (Invitrogen) were added to a 0.2-ml thin-wall PCR tube, incubated at 70°C for 10 min and immediately placed on ice. After 1 min on ice and a brief centrifugation pulse to collect contents, 4 μl of 5X First-Strand Buffer (Invitrogen), 1 μl of 0.1 M DTT (Invitrogen), 40U RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) and 0.5 µl of SuperScript™ III reverse transcriptase (RT) (200 units/µl) (Invitrogen) were added for a final volume of 20 μl. The reaction was incubated at 25°C for 5 min and at 50ºC for 1 h. Upon completion of first-strand cDNA synthesis, the reaction was heated to 70°C for 15 min to inactivate the RT and then immediately stored at -20ºC until further use.

PCR amplification of target genes was performed using 1 μl of the cDNA preparation, 1× PCR buffer (Invitrogen, USA), 3 mM of $MgCl_2$ (Invitrogen), 0.2 mM of each dNTP (Invitrogen), 1 μM of each primer (Table 2) and 1 U of Taq DNA polymerase (Invitrogen, USA) for a final volume of 25 μl. The reaction mixture was cycled through a initial denaturation at 95ºC for 5 min, followed by 35 cycles of 95ºC for 1 min, the adequate annealing temperature for 1 min (58.5ºC for *rpo*B, 43ºC for *arc*AC and 50ºC for *mle*A) and 72ºC for 1 min; a final extension step was performed at 72ºC for 5 min and thereafter cooled to 4ºC. Positive control reactions (using genomic DNA instead of RNA) and negative control reactions (using only water, as well as total RNA) were always included.

Table 2 - Primers used for gene expression analysis.

Gene	Sequence (5' - 3')	Reference
***rpo*B**	F - TGTCCGATCGAAACACCTGAAGG R - TGACGTTGCATGTTCGCACCCAT	Giacomazzi et al., 2004
***mle*A**	F - GTGCCGCTTTTTTGGATATTA R - AGCAATTTTATCTTTATAGCT	Divol et al., 2003
***arc*AC**	F - CAAGTGAGTTGTCTCGTG R - GATAAGATAGCATTGCCAC	Divol et al., 2003

PCR products were observed by gel electrophoresis on a 1% agarose gel using Tris-borate EDTA buffer and stained with ethidium bromide. The DNA bands were visualized under UV illumination and gel image was captured using a KODAK 1D system (version 3.6). Gel images were analysed using ImageJ 1.42 from the National Institute of Health (http://rsb.info.nih.gov/ij/index.htm) to obtain integrated density parameters (area × mean gray value) of the entire area of each band/amplicon. The values obtained were used to assess for differences in gene expression after

growth in the different assayed conditions. When comparing the influence pH, ethanol, malic acid, sulphur dioxide or synthetic wine on the expression profiles each gene was normalized to the housekeeping gene *rpo*B (coding for β subunit of RNA polymerase) to obtain its corresponding expression level (EL) (Bustin, 2002; Pfaffl, 2001):

$$EL = \frac{\text{Integrated density for gene X in growth assay Y}}{\text{Integrated density for } rpoB \text{ in growth assay Y}}$$

To test the reproducibility of the assays, replicates representing 10% of the samples were analysed.

Results

In a previous study, *O. oeni* strain ID57 revealed capacity to degrade arginine and produce citrulline and ornithine via ADI pathway and also to convert malic acid into lactic acid.

The influence of typical wine stresses upon *O. oeni* malolactic enzyme gene (*mle*A) and arginine deiminase gene cluster (*arc*AC) was tested by reverse transcriptase (RT)-PCR analysis. The following abiotic stresses were examined: pH, ethanol, malic acid and sulphure dioxide. The expression of these genes was also tested under conditions simulating wine environment (synthetic wine). No amplification was obtained for the water and RNA negative controls and amplicons with the expected size were obtained for the positive controls (data not shown). As expected due its housekeeping nature, the expression of *rpo*B gene was constant in all the different growth assays, whereas differences in gene regulation imposed by the different abiotic stresses were observed for *mle*A gene and *arc*AC cluster gene (Figure 1).

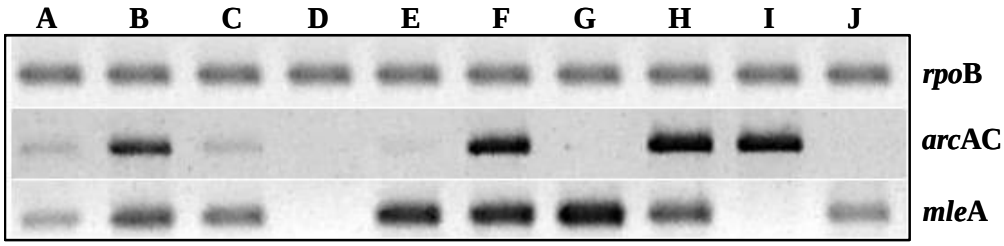


Figure 1 - RT-PCR analysis of *rpo*B, *mle*A, and *arc*AC transcripts of *Oenococcus oeni* ID57 cells. A: pH 3.5; B: pH 5.5; C: 0.1% malic acid; D: without malic acid; E: 11% ethanol; F: 14% ethanol; G: 10 mg/L SO_2; H: 20 mg/L SO_2; I: 30 mg/L SO_2; J: Synthetic wine.

Expression levels (EL) of *mle*A gene and *arc*AC gene cluster in *O. oeni* ID57 were determined in order to evaluate the effect of each stress factor. Comparing the results obtained under the different pH conditions (3.5 and 5.5), an increase on the expression of both genes associated with a pH increase was observed (Figure 2A and 2B). In this case, both genes were up-regulated in MRS pH 5.5 (assay B) and down-regulated in MRS pH 3.5 (assay A).

To determine the effect of malic acid on the expression of *mle*A gene and *arc*AC gene cluster in *O. oeni* ID57, MRS broth without malic acid or supplied with 0.1% or 0.5 % malic acid were analysed and ELs for both genes in each condition were determined and compared. An up-regulation for both genes was observed in MRS pH 5.5 supplied with 0.5% of malic acid (assay B) and down-regulation for both genes was observed in MRS pH 5.5 supplied with 0.1% malic acid (assay C). In MRS pH 5.5 without malic acid (assay D) both genes were not expressed (Figure 2A and 2B).

Expression levels under ethanol stress were evaluated in MRS broth: ethanol concentration of 11% (assay E) and 14% (assay F). The *arc*AC gene cluster was down-regulated in the presence of 11% of ethanol and up-regulated in the

presence of 14% of ethanol. The *mle*A gene was up-regulated in the presence of both concentrations of ethanol, but the up-regulation seemed higher in the presence of 14% ethanol (Figure 2A and 2B).

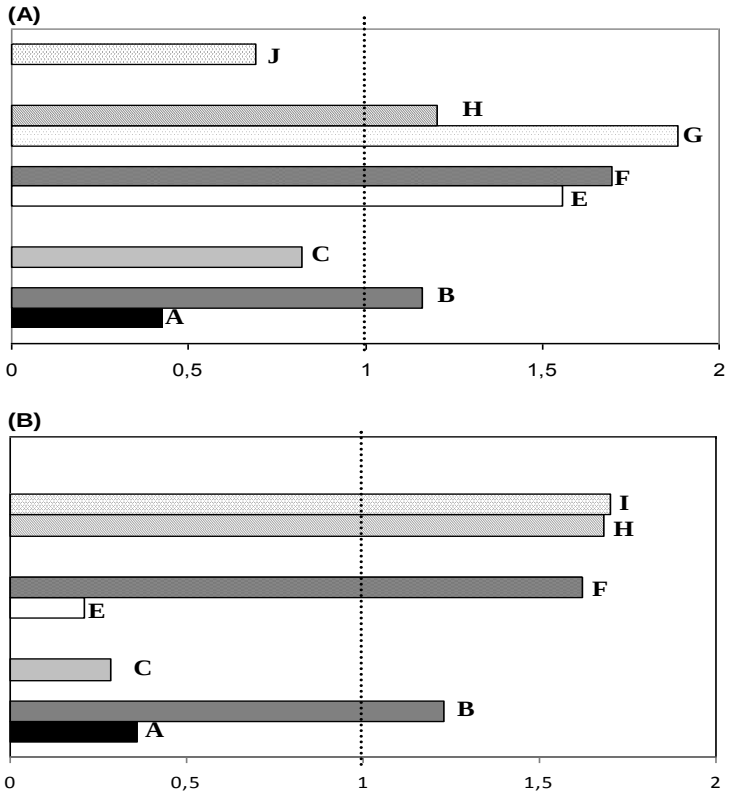


Figure 2 - Influence of pH, malic acid, ethanol and sulphur dioxide concentration and synthetic wine on the expression levels (EL) of *mle*A gene (A) and *arc*AC gene cluster (B) in *O. oeni* ID57. A: pH 3.5; B: pH 5.5; C: 0.1% malic acid; D: without malic acid; E: 11% ethanol; F: 14% ethanol; G: 10 mg/L SO_2; H: 20 mg/L SO_2; I: 30 mg/L SO_2; J: synthetic wine. ELs were normalized relatively to the housekeeping gene *rpo*B. EL = 1 corresponds to expression similar to the housekeeping gene (represented by a dotted line); EL < 1 corresponds to down-regulation of the genes relatively to *rpo*B; EL > 1 corresponds to up-regulation of the genes relatively to *rpo*B.

Sulphur dioxide effect on gene expression was also evaluated and ELs for *mle*A gene and *arc*AC gene cluster in each condition were calculated. The *mle*A gene was up-regulated in the presence of 10 and 20 mg/L of SO_2 (assays G and H, respectively), but expression level was higher at 10 mg/L of SO_2. This gene was not expressed in the presence of 30 mg/L of SO_2 (assay I). The *arc*AC gene cluster was up-regulated in the presence of 20 and 30 mg/L of SO_2, but in the presence of 10 mg/L of SO_2 was not expressed (Figure 2A and 2B).

When *O. oeni* strain ID57 was grown in synthetic wine only *mle*A gene was expressed, although down-regulated (Figure 2A and 2B).

Discussion

The fact that neither *mle*A gene or *arc*AC gene cluster are expressed in the absence of malic acid is in accordance with previous results. Under the conditions used in this study, as stated above, it was observed an increase on the expression level of both genes, *mle*A gene and *arc*AC gene cluster, associated with a pH increase, both genes being up-regulated in MRS pH 5.5 and down-regulated in MRS pH 3.5. Although cell growth is increased when the media pH is about 5 being favoured by malic acid, as the gene expression was normalised, the obtained results will not be related to higher biomass production. On the contrary, malolactic enzyme activity is higher at lower pH. Salema *et al*. (1994) described an uniport mechanism facilitating the uptake of monoanionic L-malate, at low pH and the generation of both a transmembrane pH gradient and an electrical potential gradient. Moreover, synthesis of ATP was observed in cells performing malolactic fermentation, ATP synthesis presenting the highest rates at acidic pH (Salema *et al*., 1996a, b).

The observed genes up-regulation under higher pH can only be understood as a mechanism developed by the cell to balance the decrease of the enzymatic activity. Also, as the normalisation was done versus *rpo*B gene, if this gene

was affected by the growth media pH conditions, this fact can alter the values of the calculated relative expression level of the studied genes. The effect of pH and ethanol on growth and malic acid metabolism (malolactic enzyme activity) by *O. oeni* was previously studied (Silveira *et al*., 2002, 2003, 2004; Silveira and Abee, 2009; Teixeira *et al*., 2002).

The genes up-regulation observed in presence of ethanol are in accordance to previous studies that have demonstrated an adaptive mechanism developed by *O. oeni* cells grown in presence of 8-10% ethanol, based on modification of the physicochemical state of the cells that are able to adjust their membrane permeability by decreasing fluidity at the lipid-water interface (Silveira *et al*., 2002; Teixeira *et al*., 2002; Beltramo *et al*., 2006). The composition and permeability of the cytoplasmic membrane appear to be changed and lower rates of passive proton influx were observed in ethanol-adapted cells especially at pH 3.5. These observations were in turn correlated to the high levels of ATP observed in ethanol stressed, MLF performing cells. Although 14% ethanol will induce an inhibition of enzymatic activities, the up-regulation of the genes appears as another protective mechanism developed by the cells under adverse conditions.

The SO_2 effect on the expression of both studied genes appears to be different. For *mle*A gene it follows a similar pattern to that observed for cell growth, showing a decrease on the gene expression level when SO_2 concentration increases in the culture media. A mechanism similar to that admitted for pH effect can also be considered in this case. The *arc*AC gene cluster was up-regulated in the presence of 20 and 30 mg/L of SO_2, but in the presence of 10 mg/L of SO_2 was not expressed.

Regarding the effects of synthetic wine (pH 3.2, 0.5% malic acid, 10% ethanol, no SO_2; Liu *et al*., 1995) on gene expression, down-regulation of *mle*A and no expression of *arc*AC was found. The relative level of *mle*A expression was higher than the one found for MRS pH 3.5 but lower than the one found for MRS 11% ethanol, pointing to the existence of fine-tuning mechanisms of

gene expression as an adaptive response to small variations in pH and ethanol. The absence of *arc*AC expression indicates that this additional energy-supplying mechanism is not activated by such combination of low pH and ethanol content, highlighting its importance for more adverse conditions (20-30 mg/L SO_2 and 14% ethanol). Distinct transcriptional behaviours in culture medium and wine have already been reported for *O. oeni* strains (Beltramo *et al*., 2006; Capozzi *et al*., 2010), revealing both the striking adaptability of these wine bacteria and the usefulness of expression assays as an approach to study the stress response of *O. oeni*.

The present study contributes to understanding how changes on the environmental conditions affect gene expression of *mle*A gene and *arc*AC gene cluster in *O. oeni* to allow a better knowledge regarding the selection of strains to be used as malolactic starters. Our results also highlight the importance of these studies to evaluate the transcriptional behaviour of genes that encode for enzymes with oenological relevance, as criteria in the selection of starter cultures.

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CHAPTER 5

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

5.1 General Discussion

General Discussion

Wine is an extraordinarily complex environmental matrix, consisting of exceptionally numerous compounds, that interferes with microbial growth and survival, either on a beneficial or detrimental way. Alcoholic fermentation of grape juice sugars is not the only pathway in which microorganisms are involved along wine production. Their role is also important in the conversion of L-malic acid into L-lactic acid, a process known as malolactic fermentation (MLF), generally carried out by *Oenococcus oeni*, a lactic acid bacterium (LAB). The metabolic activity of some *O. oeni* strains may give rise to undesirable compounds, such as biogenic amines (BA) and ethyl carbamate (EC) precursors.

BA are naturally occurring ubiquitous compounds resulting from the decarboxylation of the corresponding amino acids by action of microorganisms (Lounvaud-Funel, 2001). The manufacturing process of wine holds an ideal environment for BA production, because it involves available free amino acids and the potential development of selective conditions that could benefit the growth of decarboxylase-positive microorganisms and affect the activity of decarboxylase enzymes. The definition of wine quality should have in attention its BA contents mainly due to consumer protection and commercial interests. Therefore, it becomes necessary to reduce the amounts of BA in wine. As the presence of BA in wine depends on multiple complex factors, it is important to conduct critical analysis to better understand the prevention and control of the formation of these compounds. In the present study the effect of several oenological factors (winemaking region, grape variety, anti-fungal treatment of grapes, fermentation activators, malolactic starters and storage on lees) was analysed in order to evaluate their impact on the BA content in wines. The obtained data suggest that the specific winemaking region affects the amounts of amines in wine. Moreover, it was observed that wines produced in two distinct wineries from Dão region demonstrated different contents of BA,

suggesting that the grapes origin, the sanitary state, the winemaking processes and the indigenous microorganisms may change between wineries and their produced batches, even within the same region. Also, the variation among BA levels detected between wines produced from different grape varieties suggests that amines content in wine are dependent on inherent types of amino acids composition and their respective amounts in each grape variety, but also on the natural indigenous bacteria present in grapes. The analysis of anti-fungal treatment assays demonstrated that wines produced from grapes treated with anti-fungal products presented lower content of BA than those obtained from untreated grapes. This data supports the hypothesis that besides bacteria, fungal metabolic activity may influence the formation of amines in wine. Another factor that appears to contribute for the increase of BA in wines is the presence of fine lees. These fine lees are composed by residues, including proteins mainly resulting from yeast autolysis. These proteins are hydrolysed and give rise to peptides that undergo further processing into amino acids and amines. In what concerns the application of malolactic starters, the present data showed that the amounts of BA were substantially higher among wines with MLF conducted by indigenous malolactic bacteria than in wines inoculated with commercial malolactic starters, demonstrating that the use of reliable selected malolactic starters can minimize BA production.

O. oeni is an alcohol-tolerant, acidophilic LAB that plays an important role in the elaboration of wine, where it is often added as a starter culture to carry out MLF (Liu, 2002; Marcobal *et al*., 2008). Given the economic importance of this conversion, the taxonomy of this species has been studied in detail by several authors. In the present work, a new molecular method, based on the amplification of 16S rRNA gene with universal primers, followed by restriction with the endonuclease FseI, which allows the identification of *O. oeni* and its specific detection in wine, was developed. The specificity of FseI restriction for *O. oeni* 16S rRNA gene was investigated among 22 species of LAB generally

found in wine as well as their phylogenetic closest neighbours. A specific FseI recognizing sequence located at ca. 300 nt of the 5' end of 16S rRNA gene was found to be exclusive of *O. oeni* (among wine bacteria), using *in silico* comparative sequence analysis, thus ensuring the specificity of the developed method. The use of Whatman FTA cards for DNA extraction and purification is an efficient and interesting method, since samples can be easily collected at wineries by a non-specialized technician, stored at room temperature and sent in a mail envelope to the analytical laboratory for processing. This easy-to-use method, in association with the amplification of 16S rRNA gene followed by restriction with the endonuclease FseI, results in a reliable detection assay presenting a detection limit between 10^2 and 10^3 cfu/mL and a full turnaround time of ca. 8 hours. This new application ensures an efficient cost/time-saving detection of *O. oeni* in wine samples during winemaking surveillance and wine quality control.

A culture collection of 121 *O. oeni* isolated from wines of different winemaking regions (Dão, Ribatejo and Alentejo) of Portugal was characterized using phenotypic and molecular methods. M13-PCR fingerprinting analysis is a method that can be applied in the molecular typing of bacterial strains (Giraffa and Rossetti, 2004). In the present study, this method was carried out to evaluate the genetic diversity and search for underlying patterns of regional/geographical strain diversity. This study showed a high level of intraspecific genomic diversity in *O. oeni* that could be partitioned according to the geographical origin of the isolates. The M13-PCR fingerprinting analysis appears to be an appropriate methodology to study the *O. oeni* ecology of wine during MLF, as well as to trace new malolactic starter cultures and evaluate their dominance over the native microbiota.

Generally, induced MLF is recommended. However, commercial malolactic starters are not always successful under all wine conditions. The regional *O. oeni* strains are better adapted and spontaneous MLF is therefore a regular occurrence. The dangers of spontaneous MLF include a negative contribution

to flavour, production of volatile acids, increased EC and BA production. It is important, however, to distinguish potentially beneficial *O. oeni* strains. The selection of *O. oeni* strains presenting the best performances for winemaking is a complex challenge (Bou and Powell, 2005). To achieve this goal, regional *O. oeni* strains isolated previously from wines of different winemaking regions of Portugal (Marques *et al*., 2011) were characterized in terms of malolactic and β-glucosidase activity, production of BA and EC precursors and growth behaviour under distinct conditions. The obtained results showed that most of the isolated *O. oeni* strains revealed β-glucosidase activity and high levels of malolactic activity. This work shows that only some specific *O. oeni* strains were detected as capable to produce putrescine and/or cadaverine, pointing to the strain-dependent nature of this ability. Low amounts of citrulline and carbamyl phosphate were detected, corroborating the hypothesis that the capacity to degrade arginine via ADI pathway appears to be widespread among strains of *O. oeni*, although at low level.

Based on the M13-PCR fingerprinting analysis (Marques *et al*., 2011) and on the enzymatic and genetic analysis of desirable oenological characteristics of the culture collection of 121 *O. oeni* strains, a set of 26 *O. oeni* strains was selected. Additionally, 19 *O. oeni* strains isolated previously from Douro wine by Inês (2007) and six commercial malolactic starters were selected in order to assess functional behaviour in different growth conditions (pH, ethanol, sulphur dioxide, malic acid, and temperature), using “synthetic wine” and culture media, as an additional criterion to select the best and reliable *O. oeni* starters for vinification process. By applying multivariate statistics to growth data it was possible to carry out a thorough selection of the most suitable regional *O. oeni* strain for MLF, from each winemaking region of Portugal. Additionally, the multivariate statistical analysis allowed the selection of two *O. oeni* isolates from winemaking region of Dão and Douro, ID58 and AI202, respectively. These strains showed a high capability to grow in the presence of sulphur dioxide, high ethanol concentration and low pH. Moreover, they

produced low amounts of EC precursors and were unable to synthesize BA. Therefore, strains ID58 and AI202 showed to be the most promising candidate regional *O. oeni* strains to be used as malolactic starters in the wine industry. In summary, the present results highlight the importance of *O. oeni* strains screening and characterization in what concerns the production of enzymes and undesirable compounds as essential criteria for the selection of malolactic starters.

Random Arbitrarily Primed PCR (RAP-PCR) is a powerful method that can be applied to identify differentially expressed genes (Frias Lopez *et al*., 2004). In the present study, the transcriptional profiles of four Portuguese autochthonous *O. oeni* strains (AI202, ID58, Agro1 e IAL7) and two commercial malolactic starters (VP41 e PSU-1) under wine environmental conditions were analysed in order to study the effect of distinct wine matrices and to investigate the potential of this approach in the selection of starter strains. The differences observed in RNA profiles could be attributed to the distinct wine environment conditions, but some common transcripts appeared in all samples independent of the strain and wine/culture medium. These transcripts may correspond to housekeeping genes or genes associated with metabolic pathways common to growth/adaptation in wine. In the Factorial Correspondence Analysis (FCA) was possible to observe that the transcription profiles of *O. oeni* regional strains seem to be affected by the different wines, presenting high variation on the distribution of the transcription profiles. Contrarily, *O. oeni* starter strains were not as affected as the regional strains by the wine environment, showing more constrained and limited transcription profiles for each wine. The obtained results point to a clear influence of the environmental matrix in which the bacteria are cultured. These results demonstrate that transcriptional analysis can be used as a tool to evaluate *O. oeni* adaptation to a set of environmental stress factors. Thus, this method may be used as an additional tool to be explored in *O. oeni* selection. Commercial starters strains appear as having the small number of amplicons,

that in some wines and/or incubation times are even fewer than in the control MRSm laboratory medium. The distribution of the transcription quantitative patterns for each strain in the same wine at three different incubation periods was more homogeneous than the distribution of the transcription quantitative patterns across the different wines. Correlating multiple differences in the transcriptome of several *O. oeni* strains in different wines and medium conditions enables the achievement of robust information that may be used for selection of the most promising strains to induce MLF in wine. The present study developed a rapid and accurate methodology, in which the transcription profiles of *O. oeni* were analysed. The generated data showed that the transcription profiles of universal starters are not uniform when inoculated in wines from different winemaking regions of Portugal, therefore pointing to a distinct behaviour when compared to the one of the regional strains. Although the wine matrix appears to be the dominant factor in gene expression, the behaviour of each strain seems to be dependent on its gene pool. Thus, this behaviour may be associated with differential gene expression pools induced by differences in the wine matrix.

In order to evaluate the potential applicability of transcription studies in the process of malolactic starter selection, the study of the response of malolactic enzyme (*mleA*) gene and arginine deiminase (*arcAC*) gene cluster in a specific *O. oeni* strain (isolated from a Portuguese wine) under the effect of different wine stresses (malic acid, pH, ethanol and sulphur dioxide) by reverse transcription polymerase chain reaction (RT-PCR) was also performed. The *mleA* and *arcAC* showed down-regulation for low concentrations of malic acid and low pH, as well as up-regulation for high ethanol content. High levels of sulphur dioxide reduced *mleA* and increased *arcAC* expression. The observed results are here considered as adaptive responses to compensate possible inhibitory effects of the environmental conditions on the physiological activities of the bacterial cells. Intermediate down-regulation of *mleA* and no expression of *arcAC* was found in synthetic

wine, thus pointing to the existence of fine-tuning mechanisms of gene expression as well as to the relevance of transcriptional studies as criteria in the selection of malolactic starters. The present study contributes to understanding how changes on the environmental conditions affect gene expression of *mleA* gene and *arcAC* gene cluster in *O. oeni* and to a better knowledge regarding the selection of strains to be used as malolactic starters. Our results also highlight the importance of these studies to evaluate the transcriptional behaviour of genes that encode for enzymes with oenological relevance, as criteria in the selection of starter cultures.

In conclusion, the present work showed that (1) winemaking region and grape variety, anti-fungal treatment of grapes and malolactic starters are critical factors for BA production; (2) Whatman FTA cards for DNA extraction in association with 16S rRNA gene amplification and specific enzyme restriction is a reliable and easy-to-use method for the detection of *O. oeni* in wine; (3) intraspecific genomic diversity in Portuguese autochthonous *O. oeni* strains could be grouped according to the geographical origin of the isolates; (4) oenological characterization and functional behaviour under different conditions of *O. oeni* strains allowed to select four promising candidates to be used as malolactic starters; (5) contrarily to commercial starters, the regional strains showed a high variation on transcription profiles for each wine.

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5.2 Future perspectives

Future perspectives

Additional investigation is required to understand and establish limits for BA levels in commercial wines. Also, further toxicological studies should be performed in order to analyse the potential impact of these compounds in human health.

In order to identify differentially expressed genes, RAP-PCR transcripts obtained from the selected *O. oeni* strains should be cloned and sequenced.

The transcriptional analysis of the *mleA (*malolactic enzyme*)*, *bglH* (β-glucosidase) and *odc* (ornithine decarboxylase) genes and *arcAC* (arginine deiminase) gene cluster should be performed in other *O. oeni* strains under different wine conditions. This approach would be useful to understand the effect of different wine stresses in the gene expression and to further evaluate the potential applicability of transcription studies in the process of malolactic starter selection.

In further investigations the behaviour of selected regional starters strains should be evaluated in wine cellar-scale in order to validate their applicability for winemaking.

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